Food Control 147 (2023) 109577

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

4-acetaminophen (Paracetamol) levels in treated and untreated veal calves, an update

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A B S T R A C T

Recently, residues of 4-acetaminophen (paracetamol) have been found in urine of slaughter animals. To investigate its possible origin, a 7 week animal experiment was performed with 5 groups of 4 calves fed different amounts of roughage, and treated or not with 4-acetaminophen. Group 1 (control): new-born calves were fed, after the first days of colostrum, solely dairy based calf milk replacer (CMR). The animals from the other groups were 2–3 weeks of age at the start of the experiment and were fed the same CMR. Group 2 was administered oral Pracetam® 30 mg acetaminophen/kg Body Weight (BW) in the CMR, 5 days a week at alternate weeks, with maximum roughage. Group 3 was fed legal minimum roughage. Group 4 received the maximum roughage. Group 5 was administered human paracetamol pills 30 mg/kg BW once a week at weeks 2–6 with maximum roughage. The animals were housed in separate pens on slatted rubber floors without bedding. The controls were housed in a different room than the other animals. Urine was sampled once in the acclimatization period and for 5 days after treatment. At week 2, 4 and 6 at respectively 1, 2 and 3 days after treatment, one animal from groups 2 and 5 was sacrificed. An additional animal from group 4 was sacrificed at week 4, the rest of the animals at day 43. Urine, muscle, liver and kidney were analysed for residues of 4-acetaminophen and its metabolites. It appeared that controls and the low and high roughage animals had almost no 4-acetaminophen (<3 μg/L) in the urine and low levels of metabolites, whereas high roughage had at slaughter higher levels of metabolites than low roughage. Pracetam® treated animals showed high levels 4-acetaminophen (up to 130.000 μg/L) and metabolites during treatment, and lower levels (2800 μg/L) at slaughter. In the paracetamol treated animals 4-acetaminophen and metabolites were detectable during treatment (up to 130.000 μg/L) and until 7 days after treatment (110 μg/L). 4-Acetaminophen and metabolites were only present in tissues of treated animals, during treatment (11–14.800 μg/L) and a few days after treatment (7–55 μg/L). A striking effect was the decrease in concentration of 4-acetaminophen and metabolites in time during treatment in the tissues. Liver enzyme induction may provide a possible explanation for this.

1. Introduction

Since its introduction in 1950 (acetaminophen/paracetamol, NA4AP, N-acetyl-4-aminophenol) is a popular analgesic and antipyretic drug for use in humans. Paracetamol (acetaminophen) consists of mainly 4- acetaminophen and traces of its positional isomer 2-acetaminophen. Acetaminophen is also a metabolite of aniline, which is a building block in the synthesis of compounds like textiles, rubbers, pesticides and cosmetics (Love et al., 2012). In human urine acetaminophen can be found both in people that used paracetamol as well as people that have not taken paracetamol. The reason for this can be that the population is also exposed to aniline which renders free acetaminophen, its glucuronide and its sulphate in urine (Modick et al., 2014).

For production animals only Pracetam® (Ceva Santé Animale B.V., Naaldwijk Netherlands) is registered as 4-acetaminophen containing veterinary medicine for pigs. Recently 4-acetaminophen has been found in urine, kidneys and muscle from veal calves, and in urine of cattle and pigs (data Ducares). The origin of these findings is unknown, with the exception of pigs where Pracetam® is a registered veterinary medicine. Investigation of old urine samples from veal (2013–2015) revealed levels of 4-acetaminophen (15–970 μg/L sum total as 4-acetaminophen plus metabolites) (Fig. 1). It is suggested that as 4-acetaminophen (human paracetamol pills or Pracetam) is used in cows before transport to the slaughterhouse, probably to reduce the signs of pain and fever, but it is very unlikely that all animals are treated this way. Another scenario is that 4-acetaminophen is formed due to the usage of specific feed ingredients. Levels of 4-acetaminophen tend to be higher in veal calves that receive higher amounts of roughage (unpublished results, Ducares).

Ducares has analysed many veal calf urines and almost all urines were found positive for low levels of 4-acetaminophen and/or metabolites (1–350 μg/L). The only exception was urine of new-born calves in

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https://doi.org/10.1016/j.foodcont.2022.109577
Received 26 August 2022; Received in revised form 20 December 2022; Accepted 21 December 2022
Available online 26 December 2022
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the first 3 days of their life. In the urine of these animals no acetaminophen and/or metabolites above the detection limit was found. 4-Acetaminophen is not a registered veterinary medicine for cattle in Europe (EMEA, 1999). Recently WFSR performed a pilot experiment in which 6 human (500 mg) paracetamol pills were given to a calf (100 kg) using a balling gun to deliver the pills into the rumen. This led to high levels of 4-acetaminophen in the urine, which decreased to low levels within 24 h. In the first 24 h detectable levels were also found in kidney, muscle and liver (one animal treated and slaughtered after 24 h, data not published).

In a more detailed animal experiment, using veal calves as a model, we aimed to investigate the possible source of 4-acetaminophen and to evaluate the possibility to distinguish therapeutic treatment from "background" levels.

2. Materials and methods

2.1. Animals

All calves were Holstein Frisian calves (19 males and one female calf), divided into 5 experimental groups. Animals in the control group were newborn at the experimental facility (3 males and 1 female), the calves for the other groups were 2–3 weeks of age at start of the experiment.

Group 1: negative controls: 4 newborn calves were fed colostrum the first 3 days, followed by dairy based calf milk replacer (CMR) during 6 weeks. No roughage was given during the entire experimental period. The first 3 weeks the animals were housed in single boxes. The first 3 weeks they got milk in a bucket with a nipple and were fed three times a day. After 3 weeks they were housed with 2 in a pen and fed two times a day. Animal numbers 4316, 4317, 4318 and 4319.
Group 2: 4 calves, 2–3 weeks of age, were fed the same dairy based CMR (twice a day) with ad lib roughage. Every other week Pracetam® was administered orally via the milk for 5 consecutive days. Animal numbers 1097, 4662, 8587 and 9165.
Group 3: 4 calves, 2–3 weeks of age, were fed the same dairy based CMR (twice a day) with the legal minimum amount of roughage for 6 weeks. Animal numbers 3115, 3116, 3224 and 3987.
Group 4: 4 calves, 2–3 weeks of age, were fed the same dairy based CMR (twice a day) with the ad lib roughage for 6 weeks. Animal numbers 903, 5682, 6616 and 7803.
Group 5: 4 calves, 2–3 weeks of age, were fed the same dairy based CMR (twice per day) with the maximum roughage for 6 weeks. Once a week one dose of paracetamol (human pills) was administered orally. Animal numbers 720, 6980, 9301 and 2143.

2.2. Housing

All animals were housed with 2 in a pen. The pens had a rubber slatted floor without bedding. This was done to prevent the animals from eating bedding. The control group was housed in a separate room, apart from the other groups to avoid contamination (personnel changed cloths and boots between the control and the treated animals, control animals were first visited for feeding, sampling and health control). Between treatment groups there was an empty pen to prevent feed from mixing between pens. Room temperature, humidity and light was controlled and monitored daily.

2.3. Feed

The animals were fed a dairy based CMR, muesli and chopped wheat straw. The new-born calves from group 1 were fed colostrum from their own mother for the first 3 days.

2.4. Treatments

Group 1: controls no treatment, no roughage.
Group 2: maximum roughage, Pracetam® 30 mg 4-acetaminophen/kg BW orally in milk for 5 days in weeks 2, 4 and 6.
Group 3: legal minimum roughage, no treatment.
Group 4: maximum roughage, no treatment.
Group 5: maximum roughage, paracetamol human pills 30 mg/kg BW orally once a week.

The treatment schedule is depicted in Table 1.

The experiment was conducted from July until August 2019 at the animal research facilities of Wageningen University and Research (the Netherlands), in accordance with Dutch law and approved by the Central Authority for Scientific Procedures on Animals (CCD, The Hague, the Netherlands; 2016.D-0108.011).

Sampling and treatment (Table 1).

Week 1: No treatment; urine and manure was sampled from all animals.
Week 2: Treatment with Pracetam® (group 2) and paracetamol pills (group 5); urine and manure was sampled from Monday till Friday from all animals. Day 9 one animal of groups 2 and 5 was slaughtered.

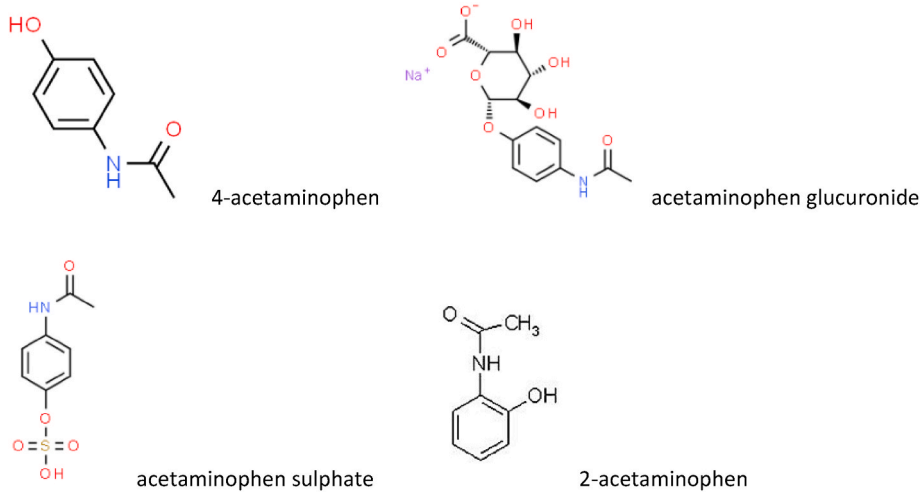

Fig. 1. 4-acetaminophen(paracetamol)), its glucuronide and its sulphate metabolite.

Table 1
Treatment and sampling schedule for the calves, group 2 receiving Pracetam® (pt) via the milk and group 5 receiving paracetamol pills (p). At week 2,4 and 6 from these groups an animal was sacrificed (†) after respectively one, two and three days of treatment for Pracetam® and after 1, 2 or 3 days after treatment with paracetamol.

day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
group	Week 1							Week 2							Week 3						
1																					
2								pt	pt	pt†	pt	pt									
3																					
4																					
5								P	†						P						
Number of samples																					
urine				20				20	20	18	18	18			18	18	18	18			
manure				20				20	20	18	18	18			18	18	18	18			
serum									2												
liver									2												
kidney									2												
muscle									2												

day	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43
group	Week 4							Week 5							Week 6							Week 7
1																						† (4 animals)
2	pt	pt	pt†	pt	pt										pt	pt	pt	Pt	pt			† (1 animal)
																		†				
3																						† (4 animals)
4			†																			† (4 animals)
5	P		†					P							P			†				† (1 animal)
Number of samples																						
urine	18	18	18	16	16			16	16	16	16	16			16	16	16	16	14			14
manure	18	18	18	16	16			16	16	16	16	16			16	16	16	16	14			14
serum			2															2				14
liver			2															2				14
kidney			2															2				14
muscle			2															2				

Week 3: Treatment with paracetamol pills (group 5); urine and manure was sampled from Monday till Friday.
Week 4: Treatment with Pracetam® (group 2) and paracetamol pills (group 5); urine and manure was sampled from Monday till Friday from all animals. Day 24 one animal of groups 2, 4 and 5 was slaughtered.
Week 5: Treatment with paracetamol pills (group 5); urine and manure was sampled from Monday till Friday.
Week 6: Treatment with Pracetam® (group 2) and paracetamol pills (group 5); urine and manure was sampled from Monday till Friday from all animals. Day 39 one animals of groups 2 and 5 was slaughtered.
Week 7: Urine and manure was sampled from all animals before slaughter.

At slaughter from each animal urine, manure, serum, liver, kidney and muscle was sampled for analysis and stored at −20 °C. Besides animal tissues, milk replacer and roughage was sampled for analysis. Serum and manure were sampled but not analysed. After collection of the whole blood, we allowed the blood to clot by leaving it undisturbed at room temperature for 30 min. The clot was removed by centrifuging at 1000×*g* for 10 min in a refrigerated centrifuge. The resulting supernatant is serum.

2.5. Materials

Paracetamol was bought at local drug stores (ETOS and Kruidvat, Netherlands).

Pracetam® 200 mg/g, REG NL 101666 (CEVA Sante Animale B.V.), was obtained from Covetrus Cuijk, Netherlands.

2.6. Liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis

Feed, urine, kidney, muscle and liver samples were analysed for 4-acetaminophen, 2-acetaminophen and its glucuronide and sulphate metabolites with LC-MS/MS. The extracts originating from feed samples were also measured on the presence of Aniline. Samples of serum and manure were not analysed. What generally applies is that each individual analysis standards are analysed added to water. These standards have subsequently gone through the full analysis process. In addition, specific internal standards were used, which automatically corrects for any losses.

2.7. Sample preparation

In accordance with applicable internal procedures, urine samples are not weighed. Urine samples were prepared by pipetting 2 mL in a polypropylene tube. Muscle, kidney and liver samples were homogenized and 5 g was weight into a polypropylene tube. 20 mL acetonitrile was added to the samples and extraction was performed by tumble-shaking the extracts at room temperature for 10 min 2 g sodium sulphate was added to the extracts, upon which the extracts were shaken for 10 min. Extracts were centrifuged at 3200 g for 10 min followed by a clean-up step using a HLB Prime column (Oasis Prime HLB 6 cc 200 mg Cat no: 186008057) where 10 mL supernatant was passed under atmospheric conditions. After this, the eluate was evaporated (45 °C with nitrogen ca 2 L per min.) until dryness in order to concentrate (Jian et al., 2019). Dried extracts were dissolved in 0.5 mL methanol/water (10/90; v/v) containing 0,1% formic acid and transferred to plastic vials with insert and stored in a freezer at −20° Celsius until analysis or directly analysed.

2.8. LC-MS/MS equipment

All samples were analysed using an UHPLC system (Agilent 1290) and a triple quadrupole mass spectrometer (Sciex 6500) operated in switching positive and negative ion mode using a turbo Ion Drive (TID) source. Analyst 1.6.3 software was used for data acquisition, Multiquant 3.0.2 software was used for data processing.

2.9. Chromatography settings

Chromatographic separation of the prepared sample extracts was achieved on an Acquity UPLC BEH C18 column (1.7 μm; 2,1 × 100 mm) column from Waters (Milford, USA) at 50 °C. The injected sample volume was 5 μL. Samples were placed prior for injection in a cooled (2–8 °C) autosampler. After each injection the injection needle and injection port were rinsed with 0.1% Formic Acid in 70% Isopropanol followed by 10% Isopropanol. The mobile phase consisted of solvents A: water with 0.1% formic acid (v/v) and B: methanol with 0.1% formic acid (v/v). A flow rate of 0.4 mL/min was used with a gradient starting at 100% A to 90% A in 4.5 min. At 8.0 min, A was decreased to 45%. The column was then flushed until 10.0 min with 100% B upon which the eluent was changed to 100% A for equilibration of the column until 11.5 min.

2.10. LC-MS/MS settings

LC-MS/MS was performed using selected multiple reaction monitoring (MRM) using Nitrogen as collision gas whereby for each compound optimised collision energy settings (CE) were used. The TID source was set at (±) 5000 V and the temperature of the TID source was set at 350 °C. For 2-acetaminophen and 4-acetaminophen MRM transitions m/z 152 > 110 (CE = 15); m/z 152 > 93 (CE = 40); m/z 152 > 65 (CE = 55); m/z 152 > 43 (49)were monitored in positive mode. For 4-acetaminophen glucuronide MRM transitions m/z 328 > 152 (CE = 15); m/z 328 > 110 (CE = 40) were monitored in positive mode. For 4-acetaminophen sulphate MRM transitions m/z 229 > 150 (CE = −22); m/z 229 > 107 (CE = −40) were monitored in negative mode. All compounds were chromatographic separated, 4-acetaminophen glucuronide eluted at 4.0 min, 4-acetaminophen sulphate at 4.6 min, 4-acetaminophen at 5.0 min and 2-acetaminophen at 6.5 min.

2.11. Detection limits

Detection limits for all four compounds were 0.5 μg/L in urine and 0.2 μg/kg in kidney. Detection limits for 4-acetaminophen, 4-acetaminophen glucuronide and 4-acetaminophen sulphate in liver were 0.2 μg/kg and for 2-acetaminophen 1.0 μg/kg. Detection limits for 2-acetaminophen and 4-acetaminophen in muscle were 0.2 μg/kg, 4-acetaminophen glucuronide and 4-acetaminophen sulphate were not monitored in muscle. All detection limits were based on signal to noise. Note that due to high background values this is an estimate.

2.11.1. UDP-glucuronosyltransferase (UGT) activity assessment

2.11.1.1. Chemicals. UDPGA tri sodium salt, p-nitrophenol, sodium hydroxide and potassium hydrogen phosphate were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Potassium chloride and potassium dihydrogen phosphate were purchased from Merck chemicals (Amsterdam, The Netherlands). Tris (Tris(hydroxymethyl)aminomethane) was purchased from Fisher Scientific (Landsmeer, The Netherlands). Acetonitrile was purchased from Actu-All (Oss, The Netherlands).

2.11.1.2. Preparation of calf liver S9. Approximately 20 g of frozen liver chunks from each calf were homogenized in ice cold Tris/KCl buffer (1.15% KCl in 50 mM Tris/HCl pH 7.4) in a precooled stainless steel blender at a ratio of 1 g tissue to 2 mL of buffer. Approximately 50 mL of the crude homogenate was transferred to 50 mL PP tubes (Greiner, Alphen aan den Rijn, The Netherlands) centrifuged in a precooled centrifuge (Universal 320R, Hettich, Geldermalsen, The Netherlands) for 25 min at 8960 rcf and 4 °C. The supernatants were collected and 500 μL aliquots were snap frozen in liquid nitrogen and stored at −80 °C. The protein concentration was determined using the Pierce BCA Protein Assay kit (Fisher Scientific, Landsmeer, The Netherlands) according to the manufacturers protocol with BSA as a standard.

2.11.1.3. Analysis of the UDP-glucuronosyltransferase (UGT) activity. The UDP-glucuronosyltransferase (UGT) activity of the liver S9 fraction of each calf was analysed by measuring rates of glucuronidation of the substrate p-nitrophenol. The incubation mixtures contained (final concentrations) 5 mg S9 protein per mL, 10 mM UDPGA and 2 mM p-nitrophenol in 100 mM phosphate buffer pH 7.4. p-Nitrophenol was prepared in DMSO and 100x diluted in the incubation mixture. The final percentage of DMSO in the mixture was 1%. Incubations were performed in low-binding Eppendorf tubes at 37 °C. The mixture was pre-incubated for 5 min and the reaction was started by addition of UDPGA. Immediately after addition of UDPGA (t = 0) and after 45, 90 and 120 min 70 μL of the mixture was transferred to a tube containing 100 μL of ice cold acetonitrile to stop the reaction. The samples were mixed thoroughly and put on ice. After all samples were collected they were allowed to warm up to ambient temperature, mixed and centrifuged at room temperature for 10 min at 14,000 rpm. 21 μL of the clear supernatant was mixed with 189 μL of 0.1M NaOH in a white, clear flat bottom 96-well microplate and absorbance was read at 405 nm in a micro plate reader (Biotek, Winooski, VT, USA). The UV absorption readouts (arbitrary units of the machine) were directly used as a marker for UGT activity. The higher the UV absorption (corresponding to unchanged nitrophenol) the lower the UGT activity.

3. Results and discussion

The animal experiment proceeded without problems. At day 10, blood was sampled from the jugular vein to determine haemoglobin levels and the calves were injected with an iron compound according to their needs. Except for some diarrhoea in the first weeks, no major health problems were encountered in the animals.

Animal samples were analysed for 4-acetaminophen, its positional isomer 2-acetaminophen and its metabolites 4-acetaminophen glucuronide and 4-acetaminophen sulphate. The feed was analysed for 2-acetaminophen and 4-acetaminophen and aniline. Samples of manure and serum were not analysed.

3.1. Feed

Low levels of 2-acetaminophen and 4-acetominophen were found in the TF-CMR. A second purchased batch from another supplier (DP-CMR) also contained some residue levels. No residues were detected in the Muesli, and in the chopped wheat straw only 2-acetaminophen was detected (Table 2). In the feed no or very low levels of aniline were found, so the formation of 4-acetaminophen from aniline cannot explain the levels found in urine of non-treated animals.

Table 2
Levels of 2-acetaminophen and 4-acetaminophen and aniline (μg/kg) in the feed; nd = not detected; * no results.

	2-acetaminophen	4-acetaminophen	aniline
Muesli V14	*nd*	*nd*	*nd*
TF-CMR	12	21	*nd*
Chipped wheat straw	15	*nd*	*
DP-CRM	9	26	*3*

3.2. Urine

The levels of 2-acetaminophen and 4-acetaminophen in the urine of the control group remained low (<6 μg/L) during the experimental period, but the levels for the sulphates and glucuronides increased over time from days 24–43 (up to 300 μg/L for sulphates and up to 320 μg/L for glucuronides). This could especially be seen in animals 4316 and 4317 on day 43.

In group 2 (Pracetam®) no detectable levels of 2-acetaminophen and 4-acetaminophen were found in the first week, but this can be explained by the high levels of 4-acetaminophen and the high dilution needed in the treatment period. So for 2-acetaminophen it was not really possible to compare the levels between the different treatment groups. Concerning metabolites in group 2 low levels of 4-acetaminophen-glucuronide and 4-acetaminophen-sulphate were detected in the first week (6–57 μg/L). During the treatment period at day 24 high levels of 4-acetaminophen (30.000–130.000 (μg/L) and its metabolites (respectively 210.000–1.4.000.000 μg/L for glucuronide and 100.000–140.000 for sulphate) were found in the urine. At slaughter, 3 days after the last treatment, these compounds could still be detected in the urine.

In group 3 (low roughage) no 4-acetaminophen was detected and only one animal presented with detectable levels of 2-acetaminophen (16 μg/L). Levels of metabolites varied between 10 and 220 μg/L for the glucuronide and between 7 and 197 μg/L for the sulphate.

In the high roughage group (group 4) 2-acetaminophen was found in all animals at day 24, the levels varied between 11 and 69 (μg/L. The levels of metabolites varied between 10 and 270 μg/L for the glucuronide and between 10 and 160 μg/L for the sulphate.

Group 5 (treated once a week with human paracetamol pills) showed no 2-acetaminophen and 4-acetaminophen during the acclimatization period, but high levels (33.000–130.000 μg/L) two days after treatment (day 24) of 4-acetaminophen and its metabolites. At slaughter, 7 days after last treatment, only low levels (16 μg/L) could be detected of 2-acetaminophen, 4-acetaminophen (110 μg/L) and its metabolites (400 μg/L glucuronide and 1.600 μg/L sulphate, Table 3).

In Table 4 the levels in the tissues are shown. No data of 2-acetaminophen were reported. It is striking to see that the levels of both 4-acetaminophen and its metabolites decreased in the tissues over time in the Pracetam® administered group, while sampling was done during treatment. An exception is day 43, which was 3 days after stopping treatment. The lower levels in the paracetamol group can be explained by the fact that the animals were treated only once a week and the sampling was after 1, 2 and 3 days of treatment.

We also looked at the sum of 4-acetaminophen and its metabolites. This is presented in Table 5.

Table 5 also shows the reduction of the levels of the acetaminophen sum over time in the Pracetam® group, although the animals were treated daily.

An explanation for these decreasing levels in the Pracetam® group can be due to that the liver function in young animals (calves in this case) is not yet fully developed (Nakagaki et al., 2018). In comparison, in humans, neonates and children have a different metabolism of acetaminophen than adults. In adults, the glucuronide is the main conjugate, in children the sulphate is more present. In an accidently overdosed neonate (child), a prolonged elimination half-life was observed (Abadier et al., 2019). This is due to the metabolic capacity of the liver which improves over time.

Another explanation can be that acetaminophen leads to enzyme induction resulting in faster clearance (Antonovic & Martinez, 2011). To investigate this we sampled S9 fractions from the livers and measured UDP-glucuronosyl transferase (UGT) activity (Fig. 3). We used a cow from the slaughterhouse as control as well as the control calves which were all slaughtered at day 43.

Fig. 2 shows the UDP-glucuronosyl transferase (UGT) activity in the livers of the calves at different ages. The first bars are from the control cow, -S9 is a control treatment without S9, and -UDPGA is the treatment without UDP-glucuronic acid. The S9-samples from the controls show slight UGT activity. In both the Pracetam® (prac) and the paracetamol (para) treated calves, the S9-samples of the 9 day old calves have a similar UGT activity as the control calves. At 24 days of age or older the treated animals show a marked increase in UGT-activity. In the Pracetam® group the activity increases with age, for the paracetamol animals this is less obvious but all activities are higher than the controls. It appears that the UGT activity is dependent on treatment and not on age, since all controls were slaughtered at day 43. Since we found these results so interesting we also analysed the low and high roughage groups as shown in Fig. 3.

As Fig. 3 shows, the enzyme activity is higher in the high roughage group. UGT activity appears to depend on the amount of roughage and/or treatment with 4-acetaminophen, not on the age of the animals.

4. Conclusion

We aimed to investigate the possible source of paracetamol in veal calf urine, liver, kidneys and meat and to evaluate the possibility to distinguish therapeutic treatment from “background” levels. This study helps to clarify potentially unexplainable residues of 4-acetaminophenol and its metabolites in food producing animals. Furthermore, the study gives important information of the presence of metabolites compared to the parent drug and the potential influence of the liver enzyme activity on residue levels. The results show that acetaminophen is present in a dairy based calf milk replacer and roughage in low levels and may contribute to the presence of 2 and 4-acetaminophen in urine in calves, which was detected in some untreated calves. Aniline levels in feed were very low or not detected and are unlikely to contribute to formation of 4-acetaminophen in urine. In treated calves predominantly 4-acetaminophen was found in high levels, whereas no or low levels of 2-acetaminohen were found. In the tissues liver, kidney and muscle only residues were found in treated animals. Further research is needed in older animals to ascertain if this is also true for animals older than 7 weeks.

CRediT authorship contribution statement

M.J. Groot: Conceptualization, Ideas, formulation or evolution of overarching research goals and aims, Methodology, Development or design of methodology, creation of models, Investigation, Conducting a research and investigation process, specifically performing the experiments, or data/evidence collection, Resources, Provision of study materials, reagents, materials, patients, laboratory samples, animals, instrumentation, computing resources, or other analysis tools, Writing – original draft, creation and/or presentation of the published work, specifically writing the initial draft (including substantive translation),

Table 3
Levels of acetaminophen and metabolites (acetaminophen-glucuronide and acetaminophen-sulphate) in urine (μg/L) in the different groups; * only 1000-fold diluted samples were measured, so no detection at a low ppb level; - no samples available.

Group	Animal	Day	2-Acetaminophen	4-Acetaminophen	4-Acetaminophen-glucuronide	4-Acetaminophen-sulphate	Group	Animal	Day	2-Acetaminophen	4-Acetaminophen	4-Acetaminophen-glucuronide	4-Acetaminophen-sulphate
1: Control calves		**day 4**					**2: Pracetam**		**day 4**				
1	4316		1	1	7	8	2	1097				12	15
1	4317		6	3	4	11	2	4662				6	7
1	4318		2			31	2	8587					28
1	4319				13	17	2	9165				17	57
		day 24							**day 24**				
1	4316				120	50	2	1097		–	–	–	–
1	4317				44	48	2	4662		*	130.000	1.400.000	450.000
1	4318				44	23	2	8587		*	30.000	210.000	100.000
1	4319				21	15	2	9165		*	70.000	330.000	120.000
		day 43							**day 43**				
1	4316				320	250	2	1097		–	–	–	–
1	4317				210	300	2	4662		–	–	–	–
1	4318		1		46	41	2	8587		–	–	–	–
1	4319				95	88	2	9165		140	2.800	2.900	7.200

Group	Animal	Day	2-Acetaminophen	4-Acetaminophen	4-Acetaminophen-glucuronide	4-Acetaminophen-sulphate	Group	Animal	Day	2-Acetaminophen	4-Acetaminophen	4-Acetaminophen-glucuronide	4-Acetaminophen-sulphate
3: Roughage min.		**day 4**					**4: Roughage max.**		**day 4**				
3	3115		1			47	4	903		1	3	10	18
3	3116				53	49	4	5682					110
3	3224					78	4	6616					50
3	3987					197	4	7803		1		27	20
		day 24							**day 24**				
3	3115				220	36	4	903		45			70
3	3116		16		60	23	4	5682		11		200	54
3	3224				150	35	4	6616		69			97
3	3987				38	15	4	7803		14			19
		day 43							**day 43**				
3	3115				15	7	4	903		–	–	–	–
3	3116				61	42	4	5682		54		140	130
3	3224				49	14	4	6616		37		220	160
3	3987				10	9	4	7803		19		270	10

Group	Animal	Day	2-Acetaminophen	4-Acetaminophen	4-Acetaminophen-glucuronide	4-Acetaminophen-sulphate
5: Paracetamol		**day 4**				
5	720					110
5	2143				21	21
5	6980					190
5	9301				120	160
		day 24				
5	720		–	–	–	–
5	2143		*	33.000	580.000	150.000
5	6980		*	130.000	2.000.000	1.200.000
5	9301		*	39.000	490.000	210.000
		day 43				
5	720		–	–	–	–
5	2143		*	110	400	1.600
5	6980		–	–	–	–
5	9301		–	–	–	–

Writing – review & editing, creation and/or presentation of the published work by those from the original research group, specifically critical review, commentary or revision – including pre-or post-publication stages, Visualization, creation and/or presentation of the published work, specifically visualization/ data presentation, Project administration, Management and coordination responsibility for the research activity planning and execution. **A. van Dijk:** Methodology, Development or design of methodology, creation of models, Software, Software Programming, software development, designing computer programs, implementation of the computer code and supporting algorithms, testing of existing code components, Not applicable, Validation, Verification, whether as a part of the activity or separate, of the overall replication/ reproducibility of results/experiments and other research outputs, Formal analysis, Application of statistical, mathematical, computational, or other formal techniques to analyze or synthesize study data, Resources, Provision of study materials, reagents, materials, patients, laboratory samples, animals, instrumentation, computing resources, or other analysis tools, Data curation, Management activities to annotate (produce metadata), scrub data and maintain research data (including software code, where it is necessary for interpreting the data itself) for initial use and later reuse. **M.J. van Baak:** Data curation, Management activities to annotate (produce metadata), scrub data and maintain research data (including software code, where it is necessary for interpreting the data itself) for initial use and later reuse, Methodology, Development or design of methodology, creation of models, Software, Software Programming, software development, designing computer programs, implementation of the computer code and supporting algorithms, testing of existing code components, Not applicable, Validation, Verification, whether as a part of the activity or separate, of the overall replication/ reproducibility of results/experiments and other research outputs, Resources, Provision of study materials, reagents, materials, patients, laboratory samples, animals, instrumentation, computing resources, or other analysis tools. **P. Boshuis:** Software, Software Programming, software development, designing computer programs, implementation of the computer code and supporting algorithms, testing of existing code components, Not applicable, Validation, Verification, whether as a part of the activity or separate, of the overall replication/ reproducibility of results/experiments and other research outputs, Formal analysis, Application of statistical, mathematical, Conceptualization, or other formal techniques to analyze or synthesize study data, Resources, Provision of study materials, reagents, materials, patients, laboratory samples, animals, instrumentation, computing resources, or other analysis tools, Data curation, Management activities to annotate (produce metadata), scrub data and maintain research data (including software code, where it is necessary for interpreting the data itself) for initial use and later reuse. **A.E. van de Braak:** Conceptualization, formulation or evolution of overarching research goals and aims, Investigation, Conducting a research and investigation process, specifically performing the experiments, or data/evidence collection, Resources, Provision of study materials, reagents, materials, patients, laboratory samples, animals, instrumentation, computing resources, or other analysis tools, Writing – original draft, creation and/or presentation of the published work, specifically writing the initial draft (including substantive translation), Writing – review & editing, creation and/or presentation of the published work by those from the original research group, specifically critical review, commentary or revision – including pre-or postpublication stages. **T. Zuidema:** Resources, Provision of study materials, reagents, materials, patients, laboratory samples, animals, instrumentation, computing resources, or other analysis tools, Writing – review & editing, creation and/or presentation of the published work by those from the original research group, specifically critical review, commentary or revision – including pre-or post-publication stages, Supervision, Oversight and leadership responsibility for the research activity planning and execution, including mentorship external to the core team. **S.S. Sterk:** Conceptualization, formulation or evolution of overarching research goals and aims, Writing – original

Table 4
Levels of both 4-acetaminophen and its metabolites (acetaminophen-glucuronide and acetaminophen-sulphate) in muscle, liver and kidney (μg/Kg).

Sample		day	number of treatments	days after treatments	Muscle (μg/Kg)			Liver (μg/Kg)			kidney (μg/Kg)		
					4-Acetaminophen	4-Acetaminophen-glucuronide	4-Acetaminophen-sulphate	4-Acetaminophen	4-Acetaminophen-glucuronide	4-Acetaminophen-sulphate	4-Acetaminophen	4-Acetaminophen-glucuronide	4-Acetaminophen-sulphate
1: control calves													
1	4316	43	–		0	0	0						
1	4317	43	–		0	0	0						
1	4318	43	–		0	0	0						
1	4319	43	–		1	0	0						3
2: Pracetam®													
2	1097	9	1	[a]	9.200	3.600	3.200	10.000	55.000	12.000	11.000	54.000	56.000
2	4662	24	2	[b]	9.600	2.600	1.100	7.600	39.000	6.400	14.800	46.000	24.000
2	8587	39	3	[c]	5.900	3.400	1.000	4.500	30.000	3.400	8.600	39.000	11.000
2	9165	43	3	[d]	0	0	0	11	29	7	7	55	53
3: roughage minimum													
3	3115	43	–		0	0	0						
3	3116	43	–		0	0	0						
3	3224	43	–		0	0	0						
3	3987	43	–		0	0	0						
4: roughage maximum													
4	903	43	–		0	0	0						
4	5682	43	–		0	0	0						
4	6616	43	–		0	0	0						
4	7803	43	–		0	0	0						
5: paracetamol													
5	720	9	1	1	1.600	830	640	510	6.100	1.700	1.300	3.500	3.000
5	6980	24	3	3	50	0	36	23	270	66	53	270	210
5	9301	39	5	5	0	0	0	5	22	4	6	23	30
5	2143	43	5	7	0	0	0	3	20	3	3	20	25

[a] At 1st treatment slaughtered after 2nd dosage.
[b] At 2the treatment slaughtered after 3rd dosage.
[c] At 3rd treatment slaughtered after 4th dosage.

Table 5
Sum of acetaminophen and its metabolites (acetaminophen-glucuronide and acetaminophen-sulphate) in tissues (μg/kg).

		DAY	MUSCLE (μg/Kg)	LIVER (μg/Kg)	KIDNEY (μg/Kg)
			Paracetamol (sum)	Paracetamol (sum)	Paracetamol (sum)
2: Pracetam®					
2	1097	9	13.000	43.000	72.000
2	4662	24	12.000	30.000	52.000
2	8587	39	8.000	21.000	34.000
2	9165	43	n.d.	29	67
5: paracetamol					
5	720	9	2.400	4.400	4.900
5	6980	24	74	190	310
5	9301	39	n.d.	18	37
5	2143	43	n.d.	14	29

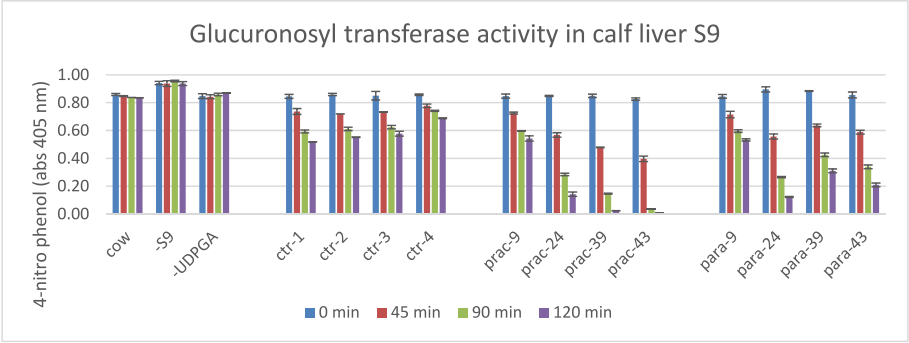

Fig. 2. UDP-glucuronosyl transferase (UGT) activity in the livers of the calves at day 9, 24, 39 and 43.

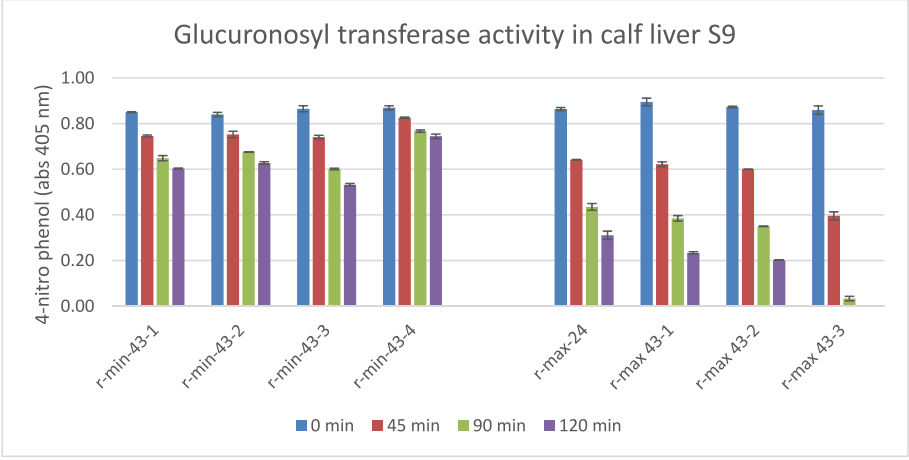

Fig. 3. UDP-glucuronosyl transferase (UGT) activity in the livers of the calves at day 9, 24, 39 and 43, minimum (r-min) and maximum roughage (r-max).

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Declaration of competing interest

Declaration of competing interest: The authors declare that they have no known competing financial interests or personal relationships that may appear to have influenced the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

This research was approved by the Netherlands Food and Consumer Safety Authority.

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