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3-NOP: ADME studies in rats and ruminating animals

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ABSTRACT

3-NOP (3-nitrooxypropanol) reduces enteric methane formation in ruminants. A series of ADME studies in rats, lactating goats and beef cattle was performed. 3-NOP was entirely absorbed from the GIT of rats: approximately 75% of the administered 3-NOP was eliminated as carbon dioxide via exhalation and approximately 20% were excreted via urine. 3-NOP is oxidized to 3-nitrooxypropionic acid (NOPA) which is then hydrolyzed to 3-hydroxypropionic acid (HPA) and inorganic nitrate, the major rat plasma metabolites. NOPA is also a plasma metabolite in beef. The metabolism of 3-NOP is fast as indicated by the negligible amounts of 3-NOP found in rat plasma 2 h after dosing. HPA is a naturally occurring metabolite. It is either metabolized into carbon dioxide and acetyl-CoA or into propanoyl-CoA, the latter serves as substrate for gluconeogenesis. Gluconeogenesis is very prominent in lactating ruminants which use propanoyl-CoA as their main carbon source. Thus, the formation of lactose from 3-NOP by lactating goats is not unexpected. Lactose was the major metabolite of 3-NOP in the aqueous phase of milk. The incorporation of 3-NOP into endogenous metabolism makes it difficult to derive a marker residue, however, conservative risk assessment could be based on the measured radioactivity in tissues.

1. Introduction

3-nitrooxypropanol (3-NOP, CASRN: 100502-66-7) has been reported to reduce the emission of the greenhouse gas methane from ruminants. A recently published meta-analysis confirms a consistently reduced methane emission in dairy cows, beef cattle and sheep at dietary concentration up to 280 mg/kg dry matter intake (Jayanegara et al., 2017). 3-NOP targets the nickel enzyme methyl CoM reductase in rumen methanogenic archaea that catalyzes the last step of the biogenic methane forming pathway (Duin et al., 2016). For lactating animals, the foreseen maximum dietary concentration is 100 mg 3-NOP/kg dry feed.

Published information on metabolism of 3-NOP is limited. Duin et al. (2016) describe the hydrolysis of 3-NOP into 1,3-propandiol in vitro in rumen fluid which is a compound of low toxicity and which is further transformed into 3-hydroxypropionic acid (HPA) a compound of the intermediary metabolism (Gingell et al., 2000).

Usually, one would aim at obtaining information from structural similar compounds with existing data before starting extensive experimental work with a particular compound. 3-NOP shares from structural point the short-chain carbon backbone with known other inhibitors of methane formation via ruminant fermentation such as the nitro-alkanes or nitro-alkanols (Zhang et al., 2018). The major difference between 3-NOP and that class of compounds is the indirect attachment of the nitrogen atom to the carbon backbone via a C—O—N-bond (see Fig. 1) as compared to the C—N-bond in the nitro-alkanes or nitro-alkanols. The toxicity and metabolism of these nitro-alkanes or nitro-alkanols was reviewed recently (Smith and Anderson, 2013). Briefly, the nitro-group of these nitro-alkanes and nitro-alkanols is hydrolyzed and inorganic nitrite is released. The carbon backbone is substrate of various endogenous metabolism pathways such as the Krebs cycle resulting in the formation of CO_2. Secondary nitro-alkanes are discussed in relation to mutagenicity/genotoxicity (Smith and Anderson, 2013). However, extensive testing on these endpoints showed no reason for concern for 3-NOP (Thiel et al., 2019). When comparing the 2D-structure of 3-NOP (3-nitrooxypropanol) with its nitro-alkanol analogue 3-nitro-propanol, the similarity score according to Dice and Tanimoto is less than 70% as evaluated by OECD QSAR

Abbreviations: AD(M)E, Absorption, distribution, (metabolism), and excretion; bw, body weight; dsm, disintegration per minute; GIT, gastrointestinal tract; (HP)LC, (High Performance) Liquid Chromatography; HPA, 3-hydroxypropionic acid; HILIC, hydrophilic interaction chromatography; KEGG, Kyoto Encyclopedia of Genes and Genomes; LSC, liquid scintillation counting; (L)LOQ, (lower) limit of quantification; MS, mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; 3-NOP, 3-nitrooxypropanol; NOPA, 3-nitrooxypropionic acid; RI, refractive index; TCA, trichloroacetic acid; TRR, total radioactive residues; UV, ultraviolett; Study 1, ADME tissue distribution and plasma kinetics in male rats; Study 2, ADE study in rats with trapping of volatiles; Study 3, Metabolite profiling in rats; Study 4, ADME study in lactating goats; Study 5, NOPA in beef cattle plasma; Study 6, inorganic nitrate kinetic profile in rats

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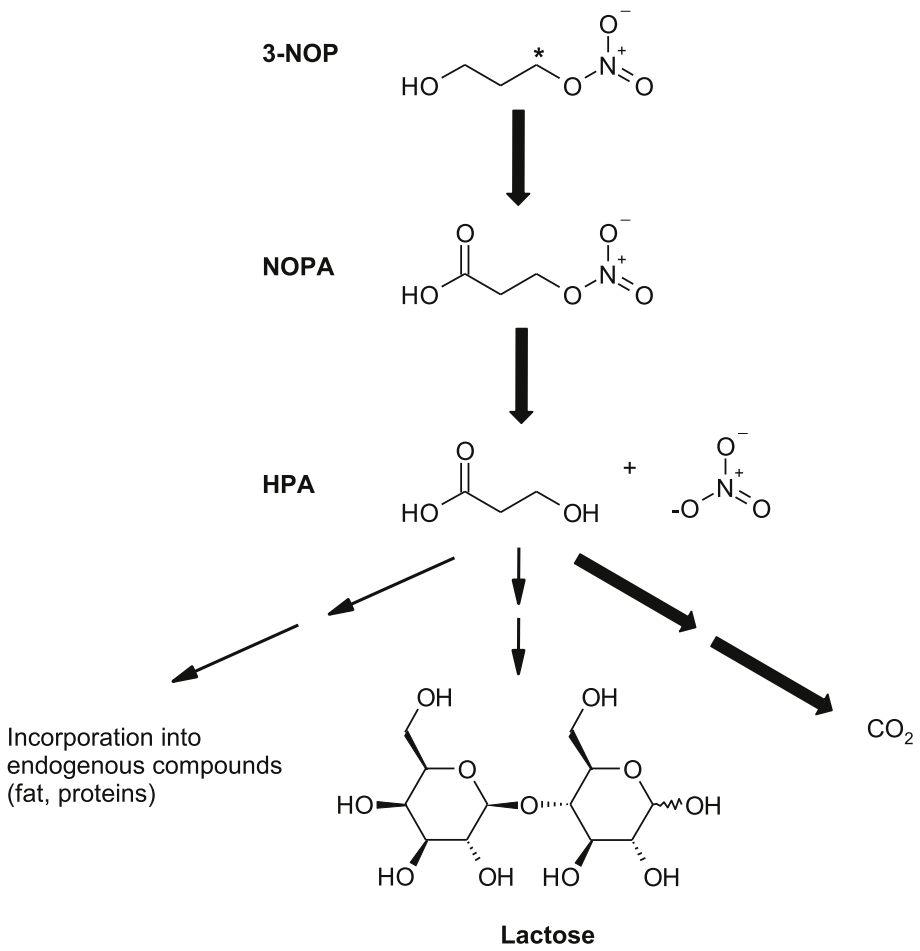

Fig. 1. Proposed metabolic pathway of 3-NOP showing the chemical structure of 3-NOP (*indicates position of the radiolabel) and the main plasma metabolites NOPA, HPA and inorganic nitrate. The bold arrow indicates the major degradation pathway.

Toolbox. Thus, 3-NOP has a low structural similarity to the nitro-alkanes and nitro-alkanols. The formation of intermediary metabolites and inorganic nitrite or nitrate was something considered during experimental testing. For regulatory purposes, we examined the ADME of 3-NOP in both laboratory animals and the target animals. Information on the metabolism and potential residue concentration in edible tissues and milk enables the preparation of a risk assessment for humans when consuming milk or edible tissues from livestock animals receiving 3-NOP. For such a risk assessment, it is also important to establish the proximity of the metabolism in the target animals and the laboratory animals because the hazard assessment is based on studies in laboratory animals (EFSA, 2012, 2017).

Three animal species were chosen for investigation, rats, lactating goats, and beef cattle. The lactating goat was chosen as a model for other ruminating livestock species thereby minimizing the amount of radioactive compound that would be needed for the study. The concentrations of NOPA as well as inorganic nitrate, the major plasma metabolite of 3-NOP identified in rats, were determined in plasma of beef cattle treated with 3-NOP. (Romero-Perez et al., 2015).

Overall, the studies reported in this paper are suitable for regulatory purposes as defined by EFSA (EFSA, 2012, 2017).

2. Materials and methods

The key chemical structures are shown in Fig. 1. The molecular weight of 3-NOP is very low (121.09 g/mol) and it lacks strong UV-absorbing structural elements. This makes the measurement of 3-NOP in biological matrices but also the analytical identification of metabolites very challenging. As a consequence, the standard ^{14}C-radiolabelling used for metabolism studies was combined with ^{13}C-labelled 3-NOP being labelled at all 3 C-atoms. The mixture of these two labelled compounds was expected to enable proper metabolite characterization, isolation, and identification using ^{13}C-NMR spectroscopic techniques in addition to mass spectrometry (MS). However, it still was difficult to identify the structures because the ^{13}C-label was in the end too diluted for NMR to be metabolite specific.

2.1. Test and control items

Three batches of 3-nitro-oxy[3–^{14}C]propan-1-ol (^{14}C-3-NOP) were custom synthetized by Selcia, UK. These had a specific activity of between 26.76 and 27.62 mCi/mmol, and a radiochemical purity between 97.2 and 99.1%. Two batches of 3-nitro-oxy[1,2,3–^{13}C]propan-1-ol (^{13}C-3-NOP) were custom-synthesized by DSM Nutritional Products with a chemical purity of 96.2% and 97.9% with an isotope enrichment of > 99%. Unlabelled 3-NOP was synthesized by DSM Nutritional Products and had a chemical purity of > 97%.

For Studies 1–3 in rats described below, the test items were formulated as a mixture of ^{14}C and ^{13}C labelled forms in molar ratios from 1/1115 to 1/1014 mol/mol in water. For the goat study (Study 4), unlabelled 3-NOP was added to the mixture of ^{13}C and ^{14}C labelled 3-NOP. The ^{14}C, ^{13}C, and unlabelled molar ratio was 1.0/59.4/30.4 for Goat 1 and 1.0/16.1/8.2 for Goat 2. 3-NOP was also used as a formulation on silica with a 3-NOP concentration of 10% (w/w). This

formulation was used in Study 5 (beef cattle, Romero-Perez et al., 2015). Unlabelled 3-NOP in water was used in Study 6 in rats.

Unlabelled NOPA was custom synthetized by DSM Nutritional Products and had a purity of 95.1%. Custom synthesized 3-Nitro-oxy [1,2,3–^{13}C]propionic acid (^{13}C-NOPA, Selcia UK) had a chemical purity of > 90%. These materials were used as described in section 6.6.2 for quantification of NOPA in beef cattle plasma.

Reference compound α-lactose monohydrate was purchased from Sigma-Aldrich; L2643 Sigma, impurities ≤4% β-lactose, BioReagent.

Reference compound D(+)-Galactose was purchased from Fluka; 48259, Assay > 99.5% (HPLC), BioChemika.

Reference compound β-D(+)-Glucose was purchased from Sigma-Aldrich; G-5250, Assay 97%, contains up to 3% α-anomer.

2.2. Other materials

Solvents used for chromatography were HPLC grade or of a similar quality.

For enzymatic hydrolysis the acid lactase Tolerase AN from *A. Niger* (DSM Nutritional Products Ltd.) was used.

2.3. Guidelines and ethical approval

The in-life phases of the rat studies were based on OECD 417 (2010) and were approved by the Swiss authorities (TVB No. AG75261, AG75267, and AG75271).

The in-life phases of the study in lactating goat were performed in an AAALAC-accredited facility according the Swiss Animal Protection Law under license no. 25296. The methods were designed to be compatible with the procedures described by EFSA (2012) and OECD 503 (2007).

Beef cattle was cared for in accordance with the guidelines of the Canadian Council on Animal Care (Romero-Perez et al., 2015).

2.4. Animals and animal housing

Male Wistar Han rats (SPF) were obtained from Janvier, Le Genest Saint Isle, France. Rats were housed in groups in Macrolon cages for acclimatization for up to 14 days. Twenty-four hours prior to administration, the rats were moved to metabolic cages (Indulab, Buchs, Switzerland) and housed individually. Rats were housed at 22 °C ± 2 °C at a relative humidity of 50–70%, a 12 h/d light cycle and 18–21 air changes per h. Animals were fed Ssniff R/M-H diet and received tap water ad libitum. When appropriate rats were cannulated at the vena jugularis. In the experiment with trapping of volatile compounds, rats were housed individually in closed glass metabolic cages (large metabolism chambers, Radleys, Essex, UK) for collection of the emitted volatiles.

The lactating Saanen Goats were supplied by a local Swiss supplier. The animals were mature, about 3–4 years old with a weight of 49.5 kg (Goat 1) and 61 kg (Goat 2). The animals were acclimatized for 7 days to laboratory conditions in a stainless-steel goat metabolism cage (W. Ehrte Versuchstiertechnik, Emmendingen, Germany). The goats received hay, ruminant feed (Provimi Kliba, Kaiseraugst, Switzerland) as well as tap water ad libitum.

Eight beef cattle were housed as described by Romero-Perez et al. (2015).

2.5. Experimental design and animal treatment

2.5.1. Study 1: ADME tissue distribution and plasma kinetics study in male rats

Four male rats (mean weight of 301 g on the day of dosing) were surgically prepared by cannulating the vena jugularis under anesthesia. The catheter (silicon tubing i.d. 0.508 mm over a PE 50 tubing i.d. 0.58 mm, total length 15 cm) was fixed in the vena jugularis and passed underneath the skin to the neck. The catheter was filled with heparinized saturated sucrose solution and closed with a metal pin. 24 h after surgery, animals received a single dose of 505 mg radiolabelled 3-NOP/kg bw (1.22 MBq) via oral gavage in water. 500 mg/kg bw was the anticipated high dose for toxicological studies. Blood samples (ca. 0.3 ml, via the cannulated vena jugularis) were collected 0.25, 0.5, 1, 2, 3, 5, 8, and 24 h post dose in addition to terminal blood 48 h post dose. Plasma was prepared immediately by centrifugation and was stored at −20 °C until analysis of TRR. Urine and feces were collected from –24–0, 0–24 and 24–48 h post dose. 48 h after dosing the animals were bled from the vena cava caudalis under isoflurane anesthesia. Tissue samples from liver, kidney, adrenal glands, spleen, heart, brain, epididymides, prostate, stomach, small intestine, cecum, colon/rectum, skin and fur, remaining carcass as well as contents of stomach, small intestines, cecum, rectum and colon were removed and stored at −20 °C until analysis.

2.5.2. Study 2: ADE study in male rats with trapping of volatiles

Two rats received a single dose of 0.93 MBq, corresponding to 506 mg ^{14}C-3-NOP/kg bw orally via gavage and were placed in the glass metabolism cages equipped for volatile trapping. The animals weighed 295 and 312 g on the day of dosing. Air was heated to 30 °C by a heat exchanger from a water bath and then continuously drawn through the cage at a flow rate of approximately 1 l/min. The air was passed through an empty trap cooled with ethanol/ice and radioactive volatiles were absorbed in a subsequent trapping flask containing 80 g 2-methoxyethanol (for collection of exhaled 3-NOP if applicable) followed by two trapping flasks in series each containing 70 g Carbosorb (for trapping exhaled carbon dioxide). The three trapping flasks were cooled in a water/ice mixture. Two portions of the trapping solvents were used for the intervals 0–8 h and 8–24 h post-dosing. Collection vessels for excreta were cooled with a mixture of solid carbon dioxide and ethanol. 24 h after dosing, animals were bled from the vena cava caudalis under isoflurane anesthesia. As described in section 6.5.1, terminal plasma, urine and feces as well as tissue were collected.

2.5.3. Study 3: metabolites in plasma, GIT, and liver in rats shortly after dosing

Three male rats (mean weight of 302 g) were treated via oral gavage with a single dose of 505 mg ^{14}C-3-NOP/kg bw (1.24 MBq per animal). 1 h, 2 h and 3 h after dosing one animal per time point was bled from the vena cava caudalis under isoflurane anesthesia and sacrificed. As described in 6.5.1, terminal plasma, urine and feces were collected. Aliquots of the terminal plasma were used for metabolite identification. The GIT was removed and separated into stomach and small intestine. The contents of stomach and small intestine were collected and frozen immediately.

2.5.4. Study 4: ADME study in lactating goats

One lactating goat (Goat 1) was gavaged once daily oral administrations of ^{14}C-labelled 3-NOP (0.38 MBq/kg bw/d) at a dose level of 4.34 mg/kg bw/d (equivalent to 111.7 mg/kg dry matter feed) for seven days. The animal was sacrificed 6 h after the last dose and terminal blood was collected. Milk was obtained prior to initial dosing and during the treatment twice daily: 0 – 8 h and 8–24 h after each daily dose. Urine was collected after the same intervals as milk. Feces was collected prior to dosing, daily during treatment and at sacrifice 6 h after the last administration. In addition to terminal blood, the edible tissues (liver, peritoneal/omental fat, muscle (shoulder and shank from fore-leg and flank, round, loin from rump), both kidneys), bile and urine from bladder as well as contents of the GIT were collected.

Based on the results of the first goat, Goat 2 was treated in a slightly different manner to achieve higher radioactivity in tissues and excreta. This goat received five daily doses via gavage of non-labelled 3-NOP followed by two daily doses of ^{14}C-labelled 3-NOP (1.04 MBq/kg bw/d) at a dose level of 3.28 mg/kg bw/d (equivalent to 101.8 mg/kg dry

feed). This animal was sacrificed 6 h after the last dose and terminal procedures were identical to those applied to Goat 1. Milk and urine were collected from 0 to 8 h, 8–24 h, and 24–30 h after the 1st radioactive dose.

2.5.5. Study 5: NOPA and inorganic nitrate plasma concentration in 3-NOP treated cattle

The experimental design is described in detail by Romero-Perez et al. (2015). Briefly, 4 mature Angus heifers per group were used. The control group remained untreated, the treatment group received 2 g 3-NOP per animal per day (corresponding to 284 mg 3-NOP/kg feed or 3 mg/kg bw/day). 3-NOP was mixed into diet (Romero-Perez et al., 2015). The feed was consumed within 4 h after feed supply. At day 29 of the study, blood samples were taken at 0, 15, 30, 60 and 180 min after providing the feed to the animals. Blood samples were also taken at the same time points from the 4 control animals that received the control feed. Plasma was prepared and NOPA concentration was determined by LC-MS. In addition, inorganic nitrate and nitrite were measured by the Griess Assay.

2.5.6. Study 6: inorganic nitrate plasma kinetics in male rats treated with 3-NOP

Five male rats (with a mean weight of 300 g on the day of dosing) received single doses of unlabelled 3-NOP either at 500 mg/kg bw (3 rats) or at 100 mg/kg bw (2 rats). Rats were cannulated as described in section 6.5.1. Blood samples were obtained 0, 0.25, 0.5, 1, 2, 3, 5, and 8 h post dose via the vena jugularis. In addition, terminal blood was obtained 24 h post dose. Plasma was prepared, and the concentration of inorganic nitrite and inorganic nitrate was analyzed.

2.6. Analytical methods

2.6.1. Liquid chromatography for metabolite characterization and isolation

An HPLC equipped with a Jasco AS-950-10 Autosampler, a Jasco PU-980 HPLC pump, Jasco MC-1510 DAD at 227 nm and a Berthold Radioflow detector LB 509 B with an YG 150 solid scintillation cell was used for HPLC Systems S1 to S4, S6, and S7.

An Agilent 1100 series HPLC system using a quaternary pump, an autosampler, a UV and a refractive index (RI) detector was used for HPLC Systems S6a and S7a.

Systems S1 to S3 were run using a Phenomenex Aqua C18, 5 µm, 125 Å, 250 × 4.6 mm (Phenomenex, Torrance, USA) column with a LiChrospher 100 RP-18e, 5 µm, 4 × 4 mm (Merck, Darmstadt, Germany) guard column. The flow was 1 ml/min.

System S1: Mobile phase A: 0.01 N HCl, pH 1.65, Mobile phase B: CH_3CN, 0 min 0% B, 5 min 0% B, 5.3 min 5% B, 20 min 5% B, 30 min 100% B, 50 min 100% B.

System S2: Mobile phase A was 10 mM ammonium formate in water at pH 3.5, mobile phase B was CH_3CN. The gradient was set as follows: 0 min 0% B, 5 min 0% B, 5.3 min 5% B, 20 min 5% B, 30 min 100% B, 50 min 100% B.

System S3: Mobile Phase A: H_2O, Mobile Phase B: CH_3CN, 0 min 0% B, 5 min 0% B, 5.3 min 5% B, 20 min 5% B, 30 min 100% B, 50 min 100% B.

System S4 was run using a SeQuant ZIC-cHILIC, 3 µm, 100 Å, 250 × 4.6 mm (Merck, Darmstadt, Germany) column with an Opti-lynx ZIC-cHILIC, 5 µm, 2.1 × 15 mm (Optimize Technologies, Oregon, USA) guard column and a flow of 0.75 ml/min. Gradient: Mobile Phase A: 5 mM ammonium formate in H_2O, mobile Phase B: 95% CH_3CN + 5% 5 mM ammonium formate in H_2O v/v.

Systems S6 and S6a were operated using a Bio-Rad Aminex HPX-87H, 9 µm, 300 × 7.8 mm (Bio-Rad Laboratories Inc., USA) column and an isocratic flow of 0.6 ml/min 0.4 mM H_2SO_4.

Systems S7 and S7a were operated using a Shiseido Capcell Pak NH2 UG80 S-5µm, 250 × 4.6 mm (Shiseido Co. Ltd., Tokyo, Japan) column with an isocratic mobile phase using 65% CH_3CN +35% H_2O v/v at a flow rate of 1 ml/min.

For systems S1 to S4, S6, and S7, peak integration was performed with the Berthold Radiostar 4.6.0.0 software. Peaks in the radioactivity chromatogram were integrated after background subtraction. The sum of integrated peaks was set to 100%. The limit of quantification (LOQ) was defined as a peak area of 1000 dpm per peak based on comparison of the integrated values with values derived from fraction collection and offline LSC measurement of a sample chromatogram.

Based on the specific activity of the individual ^{14}C-3-NOP used, the metabolite fraction or the LOQ was expressed as 3-NOP weight equivalents per extract aliquot injected onto the HPLC column. By extrapolating the aliquot volume analyzed to the volume of the total extract divided by the sample weight extracted, the metabolite concentration or LOQ in 3-NOP weight equivalents per weight of tissue or milk sample was obtained.

2.6.2. Liquid chromatography/mass spectrometry (LC-MS)

For quantification of NOPA in plasma samples from beef cattle treated with 3-NOP (Study 5) the following method was used: After protein removal and internal standard (^{13}C-NOPA) addition the supernatant was analyzed by liquid chromatography with triple-quadrupole mass spectrometry detection (LC-MS/MS) on a 4000 Qtrap from ABSciex. Unlabelled NOPA quality control and calibration samples were prepared in 5% BSA solutions and were treated analogously. The LC system consisted of a ZIC®—HILIC column (3.5 µm, 2.1 × 100 mm) and water/acetonitrile/50 mM ammonium formate/formic acid 900/50/50/1 (v/v/v/v) as mobile Phase A, and acetonitrile/50 mM ammonium formate/formic acid 950/50/1 (v/v/v) as mobile Phase B. The flow was 450 µl/min. The following gradient was used: 0 min 0.1% A, 0.9 min 0.1%A, 1 min 30% A, 1.5 min 0.1% A, 2 min 0.1% A. The detection of specific fragment ions was performed by using multiple reaction monitoring in electrospray mode with negative polarity. The LOQ was the lowest concentration used for calibration.

For metabolite identification using LC-MS, an LC-MS instrument consisting of a Rheos Allegro pump (Flux Instruments), HTS PAL autosampler with fraction collection tool (CTC Analytics), XLC 3067CO column oven (Jasco) set at 30 °C, Accela PDA (Thermo), and LTQ/Orbitrap mass spectrometer with Xcalibur V 2.1 control software (Thermo) was used. Ionization mode was negative ESI for acidic metabolites and positive ESI for sugar metabolites. Radiochromatograms were generated by fraction collection into Lumaplates and offline counting on a Topcount-NXT, model A9912V (Packard). Polar metabolites were separated under three different HILIC conditions. System MS1: Acquity BEH-Amide 10 cm × 2.1 mm column (Waters), isocratic eluent with 10% mobile phase A (5 mM ammonium acetate in water). Mobile phase B was 5 mM ammonium acetate in acetonitrile/water (95/5 (v/v =), flow rate 0.4 ml/min.

System MS2: ZIC cHILIC 15 cm × 2.1 mm column (SeQuant). Mobile Phase A: 5 mM ammonium acetate in H_2O, mobile Phase B: 5 mM ammonium acetate in acetonitrile/water (95/5 (5/5)), flow rate 0.4 ml/min. Gradient: 0 min 0% A, 2.0 min 0% A, 10.0 min 45% A, 12.0 min 45% A, 12.1 min 0% A, 15.0 min 0% A.

System MS3: ZIC cHILIC 15 cm × 2.1 mm column (SeQuant). Mobile Phase A: 5 mM ammonium acetate in H_2O, mobile Phase B: 5 mM ammonium acetate in acetonitrile/water (95/5 (v/v)), flow rate 0.4 ml/min. Gradient: 0 min 20% A, 0.5 min 20% A, 1.0 min 35% A, 14.0 min 50% A, 14.1 min 20% A, 17 min 20% A.

System MS4: ZIC cHILIC 15 cm × 2.1 mm column (SeQuant). Mobile Phase A: 5 mM ammonium acetate in H_2O, mobile Phase B: 5 mM ammonium acetate in acetonitrile/water (95/5 (v/v)), flow rate 0.4 ml/min. Gradient: 0 min 0% A, 2.0 min 0% A, 12.0 min 56% A, 14.0 min 56% A, 14.1 min 0% A, 17.0 min 0% A.

2.6.3. NMR spectroscopy

NMR was performed on a Bruker Avance III NMR spectrometer operating at 600 MHz proton corresponding to 150 MHz carbon Larmor

frequency and equipped with a cryogenically cooled 5 mm TCI probe using TopSpin 3.1 for acquisition and ACD/Labs 2012 for processing. Spectra were recorded at 298 K in deuterium oxide for lock purposes.

2.6.4. Determination of nitrate and nitrite (Griess Assay)

To determine inorganic nitrite and nitrate in plasma samples, the commercially available assay kit from Sigma-Aldrich, St. Louis, MO, USA (lot. BCBL6843V) was used and the suppliers' instructions were followed. The assay is based on the reaction of nitrite with 1-naphthylene-diamine. The reaction product is an azo dye with a maximum absorbance wavelength λ_{max} = 540 nm. The concentration of the azo dye formed was measured photometrically. The reactions were performed with sample aliquots (max. 80 µl) in 96 well microplates and the absorption was measured in a SpectraMax M5 (Molecular Devices, Sunnyvale, Ca., USA) microplate reader at 540 nm. To measure the inorganic nitrite concentration the samples were measured directly. Additionally, samples were measured with addition of nitrate reductase for determination of inorganic nitrite and nitrate concentration as sum. Control experiments showed, that the colorimetric assay was sensitive to inorganic nitrite or nitrate. However, substances with covalently bound nitrate groups did not interfere with the method. It was also shown that after spiking and incubating control plasma with the nitrite standard only small amounts of the nitrite were immediately oxidized to nitrate. The remaining nitrite was stable for up to 24 h at room temperature.

2.6.5. Determination of TRR in excreta, tissues and fluids

Studies 1 to 3:

About 250 mg aliquots of blood, erythrocytes, feces and carcass homogenate, cecum and colon content were placed in a Combusto Cone on a Combusto Pad (Packard Biosciences, USA). Moist samples were air dried over night at room temperature before combustion in a Model 307 Oxidizer (Canberra Packard, USA). The $^{14}CO_2$ was collected in 8 ml Carbosorb E liquid. The radioactivity was determined after addition of 14 ml Permafluor by liquid scintillation counting. The limit of quantification was set to be three times the background determined as a combusted blank sample.

About 250 µl aliquots of urine, plasma and up to 2 ml of extracts were added to 10 ml Ultima Gold scintillation cocktail and counted directly in a Tri-carb 2500 TR (Canberra Packard, USA) liquid scintillation counter using the Transformed Spectral Index of the External Standard Spectrum (tSIE) method for quench correction. About 250 mg aliquots of all organ and tissue homogenates were dissolved in 4 ml Soluene 350 at 50 °C for 4–18 h. After addition of 0.5 ml 4N HCl and 15 ml Irgasafe Plus the radioactivity was determined by LSC. Background values were measured with each sample sequence using the respective scintillation mixture without any sample. The limit of quantification was set to be three times the background determined with blank scintillator.

Goat study:

Radioactivity was determined on Packard liquid scintillation counters (e.g. TRI-CARB 2500 TR or 2900 TD) using the Transformed Spectral Index of the External Standard Spectrum (tSIE) method for quench correction. About 100 µl aliquots of urine, 200 mg plasma and 200 mg milk aliquots were directly counted in 10 ml Irgasafe Plus scintillation cocktail. About 200 mg samples of the homogenates of organs and tissues were digested with 2 ml Solvable tissue solubilizer at 50 °C for about 24 h. After cooling, the samples were measured with 10 ml Irgasafe Plus. Samples of approximately 200 mg whole blood were bleached with 0.25 ml H_2O_2 (30%) after addition of 1 ml Solvable and 0.5 ml isopropanol. After incubation for 10 min at room temperature and 30 min at 40 °C the samples were cooled and measured in 10 ml Irgasafe Plus.

2.6.6. Metabolites in plasma, stomach and small intestine contents, liver, and milk

Study 3:

15 µl TCA solution (1 g/ml) was added to 150 µl rat plasma. The sample was cooled for 15 min in the refrigerator and centrifuged. The supernatant was removed and analyzed by System S2.

To 0.5 g of the rat stomach content, 0.5 ml 10 mM ammonium formate in H_2O was added and acidified with formic acid to pH 3. The sample was homogenized for 10 min in an ultrasonic bath, followed by homogenization for 5 min on a Vortex mixer. The homogenate was centrifuged, and the extract was analyzed by System S2. The radioactivity in the pellet was analyzed by combustion analysis (see 6.6.5).

3.5 g of the small intestine content was homogenized for 1 min with a Polytron homogenizer, 10 min in an ultrasonic bath and 5 min with a Vortex mixer. The suspension was centrifuged, and the supernatant was analyzed by System S2. The radioactivity of the non-extractables was determined by combustion analysis.

2.3 g rat liver was extracted in 4 steps with 1.5 ml water, followed by 1.5 ml water, 3.5 ml water and 4 ml acetone. The suspensions were homogenized for 1 min with a Polytron homogenizer, 10 min in an ultrasonic bath and 5 min with a Vortex mixer. After each homogenization step, the sample was centrifuged, and the four supernatants were removed from the pellet. The first two extracts were combined and analyzed by System S2.

Study 4:

To 2 g aliquots of goat milk from interval 0–8 h and 8–24 h after the 6th dose 350 µl TCA solution (1 g/ml) was added. The sample was mixed and centrifuged. The aqueous phase was carefully removed from the pellet and the floating solid layer. The solid residues were dissolved in tissue solubilizer and analyzed by LSC. A 250 µl aliquot of the aqueous phase was analyzed by system S2.

2.6.7. Metabolite isolation and identification

2.6.7.1. Metabolites in plasma and GIT contents of rats (Study 3). To 200 µl plasma from the animal sacrificed 1 h post dose, 20 µl TCA solution (1 g/ml) was added and the sample was cooled and centrifuged. The supernatant was removed, and the radioactive metabolites were separated by System S2 (Fig. 4) and the two major metabolite fractions M3 and M6 were collected, re-purified by System S3 and analyzed by LC-MS system MS1.

A 0.5 g aliquot of the stomach content from the animals sacrificed 1 h and 3 h post dose was suspended in 0.5 ml 10 mM aqueous ammonium formate and acidified with formic acid to pH 3. After homogenization followed by centrifugation the supernatant extract was removed. The pellet was re-extracted with 1 ml 10 mM aqueous ammonium formate and the resulting supernatant extracts were combined. This combined extract was separated by System S2 resulting in one major peak which was co-chromatographed with ^{14}C-3-NOP.

A 1.5 g aliquot of small intestinal content (including water rinse) from the animal sacrificed 2 h post dose was homogenized and after centrifugation the supernatant was acidified with 10 µl formic acid and the solids were removed by centrifugation. The resulting supernatant was separated by System S2 into metabolite fractions which were analyzed by LC-MS systems MS2 and MS4.

2.6.7.2. Metabolites in milk (Study 4). For isolation of the major goat milk metabolite M13 (see Fig. 6), 7 g of the milk sample collected 0–8 h after the 6th dose was worked-up and fractionated. After precipitation of the milk fat and proteins with 0.7 ml TCA solution (1 g/ml) the aqueous phase was chromatographed in 10 repetitive injections in System S3. The major radioactive peak from the individual runs was collected and combined into one sample, which was reduced in volume in a rotary evaporator. The resulting concentrate was purified in one run using System S3. The major radioactive peak was collected, re-purified on System S1 and collected. The collected peak was chromatographed using System S4. A highly retained symmetric

radioactive peak was obtained which was collected and re-chromatographed using System S1. The resulting fraction corresponding to metabolite M13 was analyzed by ^{13}C-NMR and LC-MS (System MS3).

A second similar work-up procedure was used to isolate higher amounts of the metabolite fraction M13. The isolated metabolite fraction was analyzed using System S6, S6a and S7a.

The isolated metabolite Fraction M13 was diluted with 4.5 ml 0.1 M sodium acetate pH 4.5 and a solution of 720 ALU (acid lactase units) Tolerase AN (acid lactase) in 0.9 ml of the same buffer was added. This mixture was incubated for 2 h at 37 °C. The resulting sample was analyzed using System S4, S6a, and S7a.

The isolated fraction after lactase treatment was desalted using System S7, any remaining enzyme was precipitated after addition of TCA solution to a final concentration of 10% (w/v) and the sample was further purified by chromatography (System S2). The purified fraction was analyzed using System S6 and by ^{13}C-NMR and LC-MS (System MS3).

3. Results

3.1. Excretion balance in rats and goats

The recovery of radioactivity in Studies 1 and 4 was low (approximately 20–30%, see Table 1). To investigate whether this was due to exhalation of volatile molecules such as the parent compound and/or metabolites such as carbon dioxide, Study 2 was conducted in closed metabolism cages for collection of expired air. Most of the orally applied radiolabel was eliminated via exhaled air as carbon dioxide (approximately 75% within 24 h). Indeed, most of the exhaled radioactivity was recovered within 8 h (approximately 65% of the applied radioactivity). These results indicated extensive metabolism of 3-NOP.

Rats excreted 18% of applied radioactivity within 48 h via urine. Fecal excretion was minor (1–2% of the applied radioactivity). Thus, a minimum of 95% of the applied radioactivity was systemically available i.e. absorbed from the GIT. The levels of radioactivity in the tissue and the gastrointestinal contents shortly after dosing were higher than the levels determined at the later sampling time points. 24 or 48 h after dosing, approximately 6–8% of the applied radioactivity remained in organs and tissues of rats.

Lactating goats excreted between 4.5 and 6.4% of the total applied radioactivity via milk. The concentration of radioactivity in milk varied between 6.5 and 26.6 µg 3-NOP eq./g. There was no increase in radioactivity concentration in milk with time (see Fig. 2). Urinary and fecal excretion were about 3.5 and 1.9% of the applied radioactivity, respectively (see Table 1). The radioactivity levels in the tissues of Goat 1, 6 h after the last dose, were equivalent to 19.47 mg 3-NOP eq./kg liver, 9.39 mg eq./kg kidney, 2.05 mg eq./kg muscle, 0.80 mg eq./kg fat, and 3.25 mg eq./kg blood.

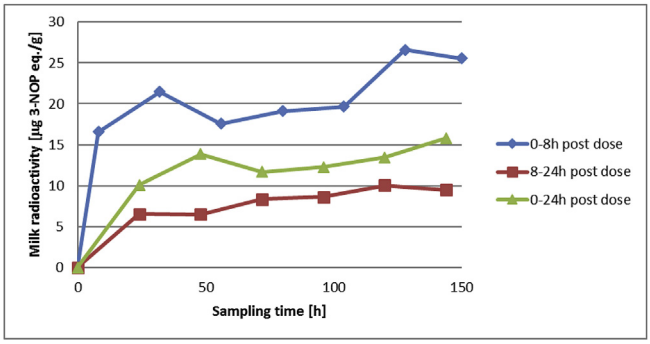

Fig. 2. Concentration of radioactivity in milk over time from Goat 1 (treated daily for 7 days with ^{14}C-3-NOP (Study 4)). The last time point is 6 h after the last dose i.e. at sacrifice.

3.2. Plasma kinetics and plasma metabolite identification in rats

In Study 1, 4 male rats were treated with 500 mg [^{14}C]-3-NOP/kg bw once orally via gavage and bled after 0.25, 0.5, 1, 2, 3, 5, 8, and 24 h post-dose. Absorption of radioactivity into plasma was fast: The radioactivity in plasma reached a mean maximum concentration of 4771 nmol 3-NOP equivalents/g (592 µg/g) after 1 h and decreased to 214 nmol 3-NOP eq./g (27 µg/g) after 48 h (see Fig. 3).

To characterize the plasma radioactivity further, Study 3 was conducted where animals were sacrificed shortly after dosing (1 animal each 1, 2, and 3 h after dosing) for collection of terminal blood. After protein precipitation with TCA 81%, 80% and 54% of the plasma radioactivity remained in the respective supernatant. Two major metabolites were found in this plasma fraction using System S2: M6 and M3 (Table 2, Fig. 4).

M6 and M3 were isolated and identified by LC-MS system MS1 as the oxidation product 3-nitrooxy-propionic acid (NOPA) and M3 as its

Table 1
Radioactivity balance and tissue distribution after oral gavage doses of ^{14}C-3-NOP to male rats and lactating goats.

	Study 1 (Rat, N = 4)	Study 2 (Rat, N = 2)	Study 3 (Rat, N = 1)			Study 4 (Goat, N = 1)	
Dose [mg/kg bw]	505	506	510	499	505	4.3	3.3
No. of Oral Gavage Doses	1 × ^{14}C	1 × ^{14}C	1 × ^{14}C	1 × ^{14}C	1 × ^{14}C	7 × ^{14}C	5 × ^{12}C plus 2 × ^{14}C
Study Duration [h]	48	24	1	1	1	174	174
Necropsy tissue/blood collection [h after the last dose]	48	24	1	2	3	6	6
Radioactivity [% of dose]							
- Urine	17.94	9.76	1.76	13.74	5.90	3.50	1.97
- Feces	2.32	1.24	n.s.	n.s.	0.03	1.90	0.71
- Cage Wash	0.36	0.38	0.00	3.77	1.58	0.03	0.02
- Expired Air	n.c.	77.44	n.c.	n.c.	n.c.	n.c.	n.c.
- Total[1)]/Edible[2)] Tissues	5.53	7.75	57.46	41.73	19.18	4.98	8.99
- Total Gastrointestinal Content[3)]	0.23	0.48	15.89	9.28	29.20	1.14	14.87
- Skin and Fur	1.61	2.26	13.26	9.14	2.50	n.c.	n.c.
- Milk	n.a.	n.a.	n.a.	n.a.	n.a.	6.42	4.48
Recovery	*27.99*	*99.31*	*88.37*	*77.66*	*58.39*	*17.97*	*31.04*

1): liver, kidney, adrenal glands, spleen, heart, brain, epididymides, prostate, stomach, small intestine, cecum, colon/rectum, remaining carcass.
2): liver, peritoneal/omental fat, muscle (shoulder and shank from fore-leg and flank, round, loin from rump.
3): contents of stomach, small intestines, cecum, rectum and colon.
n.c.: not collected n.a. not applicable n.s. no sample.

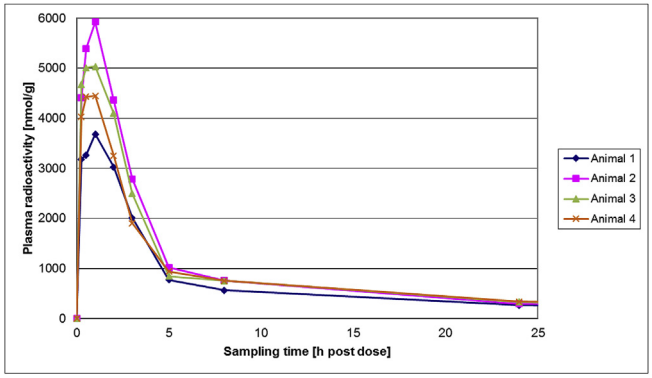

Fig. 3. Kinetics of plasma radioactivity after a single oral dose of ^{14}C-3-NOP (nominal 500 mg/kg bw) to male rats (Study 1). Individual results are shown.

hydrolysis product 3-hydroxypropionic acid (HPA). MS-data are shown in the supplemental information. However, the parent compound M7 (3-NOP) was only seen in minute amounts 1 h after dosing at 1% of the plasma radioactivity. Each of the fractions M2, M4, and M5 accounted for less than 3% of the plasma radioactivity (see Table 2).

To investigate whether the cleavage of the nitrate ester group in 3-NOP and NOPA results in inorganic nitrate as postulated, Study 6 was conducted where inorganic nitrate and inorganic nitrite were measured using the Griess Assay. After dosing of 500 and 100 mg 3-NOP/kg bw the nitrite plasma levels did not differ from the background levels and remained mostly below 20 nmol/ml (data not shown). The inorganic nitrite plus nitrate levels in plasma quickly increased from the background level of about 46 nmol/ml (Fig. 5) to a mean maximum concentration Cmax of 4853 nmol/ml and 1213 nmol/ml 1–3 h after dosing 500 and 100 mg 3-NOP/kg bw, respectively. The concentrations declined rapidly, and background levels were reached 24 h post dose.

3.3. Metabolites in liver, stomach and small intestine content of rats

In the animals sacrificed 1 h and 3 h post dose (Study 3) the major radioactive compound in the stomach content was intact 3-NOP, representing 84% and 87% of the radioactivity in the extract, respectively. This indicates that 3-NOP is very stable under the stomach conditions.

In the small intestinal content, only minor amounts (< 4%) of the radioactivity in the extract was intact 3-NOP. The major metabolite fractions were HPA (M3) and M5.

In the liver, the non-extractable fraction increased from 12% to 45% of the liver radioactivity from 1 h to 3 h post dose. No 3-NOP was detectable (LOQ ≤ 1000 ng/g). Due to the low concentration of radioactive metabolites and the high matrix load in the extracts it was not possible to attribute all metabolite fractions to known structures.

3.4. Metabolites in milk from lactating goats

Milk samples from Study 4 were separated into milk fat, milk proteins and aqueous phase (see section 6.6.6). For Goat 1, 75% and 59% of the milk radioactivity was recovered in the aqueous phase after fractionation of the milk collected 0–8 h and 8–24 h after the 6th dose, respectively. The remaining radioactivity was associated with the milk fat and protein fraction. Comparable results were obtained with milk samples from Goat 2: 77.6–84.2% of the milk radioactivity remained in the aqueous phase.

The aqueous phase (Goat 1) was analyzed by System S2 showing one polar radioactive metabolite. No exogenous substance i.e. neither 3-NOP nor NOPA was found in Goat 1 milk with a limit of quantification (LOQ) of 0.82 (0–8 h post dose) and 0.43 µg 3-NOP eq./g (8–24 h post dose). A representative chromatogram is shown in Fig. 6. For Goat 2, also no peak was detected at the retention time for the two nitrate esters 3-NOP and NOPA with an LOQ of 0.21 µg 3-NOP eq./g.

The only radiolabelled metabolite fraction (Fr1, M13, in Fig. 6) in the aqueous phase of milk was isolated as described in section 6.6.7.2 and analyzed by ^{13}C-NMR and LC-MS System MS3. The structure was identified as lactose (M13). The MS- and NMR-data can be found in the supplementary information. Fraction M13 was also chromatographed using Systems S6 and S6a confirming lactose based on its retention time. As lactose is a disaccharide composed of glucose and galactose, it was expected that treatment with lactase would produce a shift in the retention time. Thus, this isolated metabolite fraction was treated with lactase and the resulting sample was chromatographed using Systems S6a. The RI signal in this chromatogram showed a shift in retention time to galactose and glucose. 99% of the lactose was cleaved under the conditions used. Analysis using System S7a confirmed the shift of retention time towards glucose and/or galactose. The formation of glucose and galactose was further confirmed using Systems S6 and S7 with radio-detection. Two radioactive peaks with retention times identical with galactose and glucose were detected (see Fig. 7). The area of both radioactivity peaks was similar, indicating that the radiolabel was incorporated in galactose and glucose to a similar extent. The analysis by ^{13}C-NMR and LC-MS using System MS3 further confirmed that the radiolabel was incorporated in the galactose and glucose moiety of lactose (see supplementary information).

3.5. Concentration of NOPA and nitrate plus nitrite in beef plasma samples

The baseline nitrite plasma level in the control animals was approximately 2 nmol/ml and no increase was observed in the treated animals. The nitrate plus nitrite plasma levels were in the range of 10 nmol/ml and only a very small increase (if any) was observed due to feeding 3-NOP at a dose of 3 mg/kg bw/d (data not shown).

The NOPA plasma concentrations were below 0.04 nmol/ml (LLOQ) in the control samples and in all samples prior to feeding the 3-NOP supplemented diet. The NOPA plasma concentrations kinetic profiles from the treated animals varied considerably between the individual animals in the range of 0.17–1.53 nmol/ml (Fig. 8). NOPA concentrations were below the LLOQ before the next feeding.

4. Discussion

The metabolite patterns in plasma, stomach contents and in the small intestine contents in male rats after a single oral dose of 500 mg 3-NOP/kg bw showed the metabolism of 3-NOP prior to uptake in the GIT. Negligible amounts of 3-NOP were found in plasma 1 h after dosing even at the high oral dose of 500 mg/kg bw. However, plasma obtained at earlier time points after dosing was not analyzed and the presence of 3-NOP cannot be excluded entirely based on the data described. Distinct concentrations of 3-NOP in plasma was confirmed at earlier timepoints in rats treated with high dose levels of 800 mg/kg bw (Thiel et al., 2019).

There was a fast and almost complete uptake of 3-NOP associated radioactivity. Most of the orally applied radioactivity was exhaled as $^{14}CO_2$ (approximately 75%) by rats treated with high oral dose of 500 mg/kg bw indicating that 3-NOP is extensively metabolized. Urinary excretion of radioactivity accounts for approximately 20% of applied radioactivity 48 h after dosing. Such extensive metabolism into CO_2 and the formation of compounds entering the intermediary metabolism was described for some of the nitro-alkanes and nitro-alkanols as well (Smith and Anderson, 2013) and is thus not surprising.

The major plasma metabolites found in rats are 3-nitrooxypropionic acid (NOPA), its hydrolysis products 3-hydroxypropionic acid (HPA) and inorganic nitrate. Based on this preliminary Study 3 with limited number of animals per time point, NOPA decreased from about 50% to 5% of the plasma radioactivity from 1 h to 3 h post dose and HPA increased from 25% to about 50% from 1 h to 2 h post dose (see Table 2).

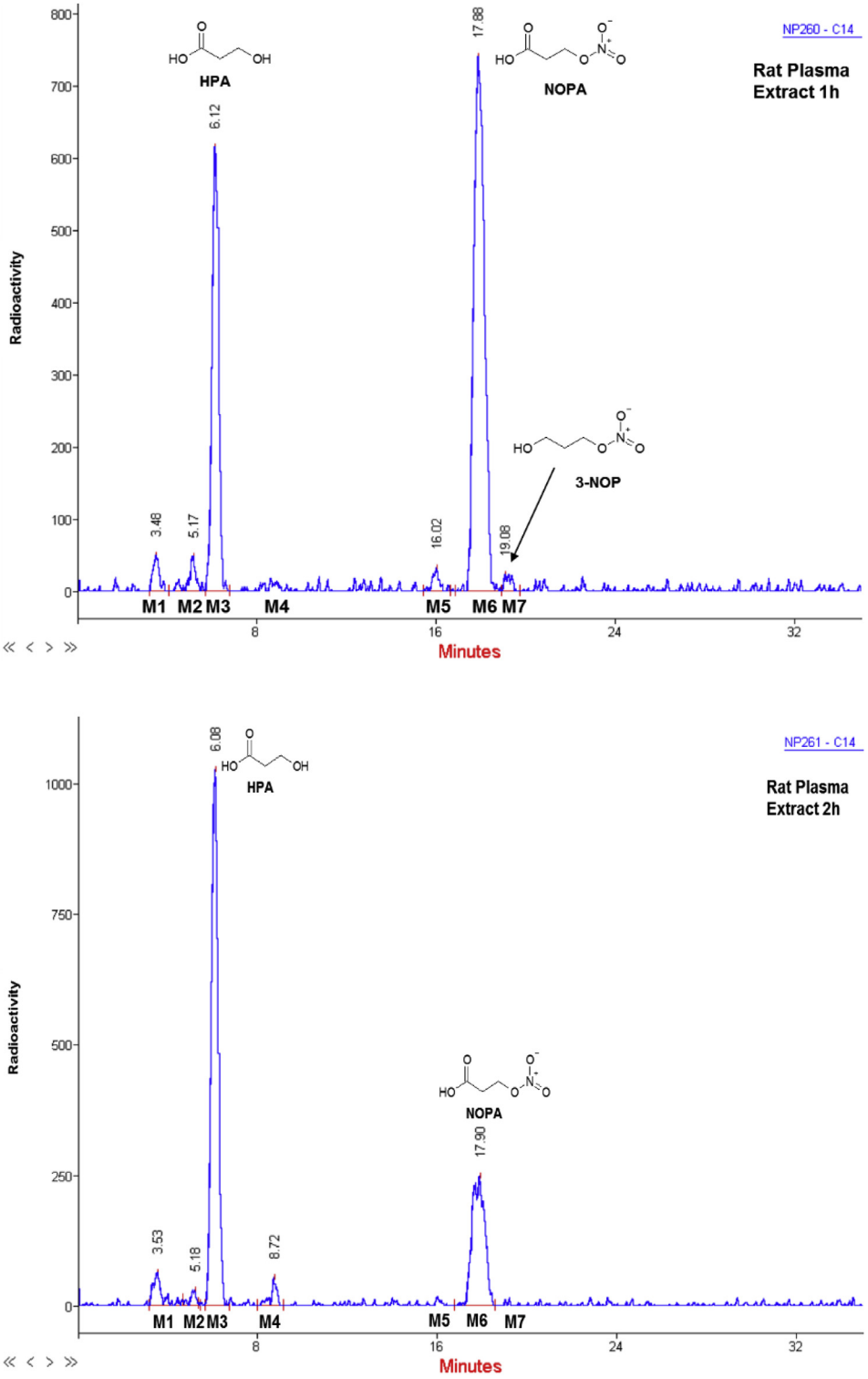

Fig. 4. Radio-HPLC chromatogram of rat plasma extract (Study 3) on HPLC system S2, 1 h post dose (upper panel) and 2 h post dose (lower panel). For peak quantification see Table 2.

Fig. 4 shows the respective chromatogram of plasma from the rats sacrificed after 1 h and 2 h. Such qualitative statements seem ambiguous in consideration of the number of animals per time point used in this study. However, the kinetic profiles of the parent compound 3-NOP and its metabolites at various time points after dosing were published earlier (Thiel et al., 2019). After a fast increase of NOPA in plasma with a Tmax of 1 h, a decrease of NOPA in plasma is observed down to about 1% of the peak concentration after 3 h. HPA concentration steadily increased up to 2 h after dosing (Tmax). The observed concentrations of HPA 1 and 3 h after dosing are approximately 30–50% of the peak concentration.

Nitro-alkans are described to release inorganic nitrite (see review of Smith and Anderson, 2013) which is further oxidized to nitrate. 3-NOP and/or its metabolite NOPA, however, release inorganic nitrate and did not change the plasma nitrite concentration.

Treatment of beef cattle with 3-NOP also resulted in distinct NOPA

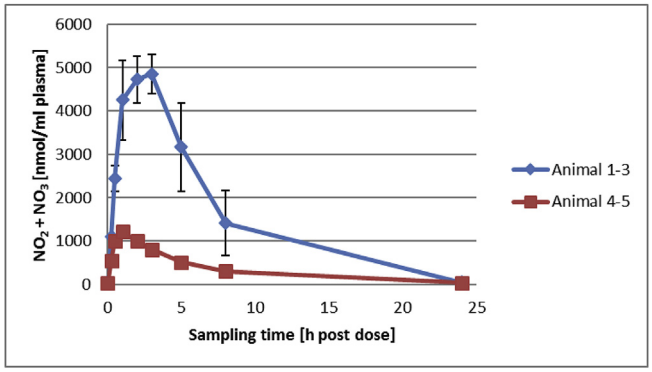

Fig. 5. Nitrate plus nitrite plasma kinetics in male rats after single oral doses of 500 mg/kg bw (blue) or 100 mg 3-NOP/kg bw (red). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

concentrations in plasma though at much lower concentrations due to lower dose levels used. The high variation in the beef samples in terms of NOPA concentration most probably reflect the differences in feed and compound uptake shortly before the plasma sampling time point. In Study 1 where rats were dosed by gavage with ^{14}C-3-NOP a very fast uptake of radioactive residues into plasma was observed with a T_{max} at approximately 1 h even at a high dose level of 500 mg/kg bw. This fast kinetics of radioactivity may explain the rapid increase and the high variability of NOPA in beef plasma. NOPA concentrations were below the LLOQ before the next feeding due to the quick elimination from beef plasma.

The low radioactivity recovery also observed in the study using lactating goats indicates that 3-NOP is also extensively metabolized to CO_2 in the target species. Only very small amounts of radioactivity were excreted via urine and feces (approximately 5% of the applied radioactivity).

The only ^{14}C-radiolabelled metabolite in milk was identified as lactose confirming a very high degree of 3-NOP breakdown and incorporation of the components into endogenous compounds. The incorporation of the radiolabel into endogenous compounds is also supported by the presence of non-extractable residues. Neither 3-NOP nor its oxidation product NOPA were detected (LOQ ≤ 200 ppb) in milk from a lactating goat treated with approximately 4 mg/kg bw, which represents the highest foreseen dietary dose of 3-NOP for lactating animals. Therefore, only minute amounts of xenobiotic substances (i.e. organic nitrate esters), if any, originating from administration of 3-NOP are expected in milk. It is also considered unlikely that such organic nitrate ester compounds will be present in edible tissues. Based on the results presented it is concluded that the majority of 3-NOP (a primary alcohol) is oxidized to NOPA most likely by alcohol dehydrogenase/aldehyde dehydrogenase enzymes in the intestine and liver. NOPA is present in the circulation and further cleaved into HPA and inorganic nitrate (Fig. 1). The parent compound 3-NOP entering the circulation will be quickly metabolized.

HPA is a natural constituent in the body (Wilson et al., 2017) and is designated as Kyoto Encyclopedia of Genes and Genomes (KEGG) compound C01013. In mammalian cells, HPA is a metabolite of the amino acid beta-alanine and pyrimidine DNA-bases. HPA can either be oxidized to malonate semialdehyde or to 3-hydroxypropanoyl-CoA (Wilson et al., 2017; Den et al., 1959).

Malonate semialdehyde is either further transformed into acetyl-CoA thereby liberating carbon dioxide (Kedishvili et al., 2000) or it can be used for the biosynthesis of fatty acids (see KEGG pathway map00410) with malonyl-CoA as intermediate.

3-Hydroxy-propanoyl-CoA is transformed into propanoyl-CoA (see KEGG pathway map00640) which is metabolized into succinyl-CoA an intermediate of the Krebs cycle. The Krebs cycle has a central role in the metabolism of aerobic cells. The Krebs cycle delivers but also consumes endogenous building blocks for the synthesis of amino acids, fatty acids

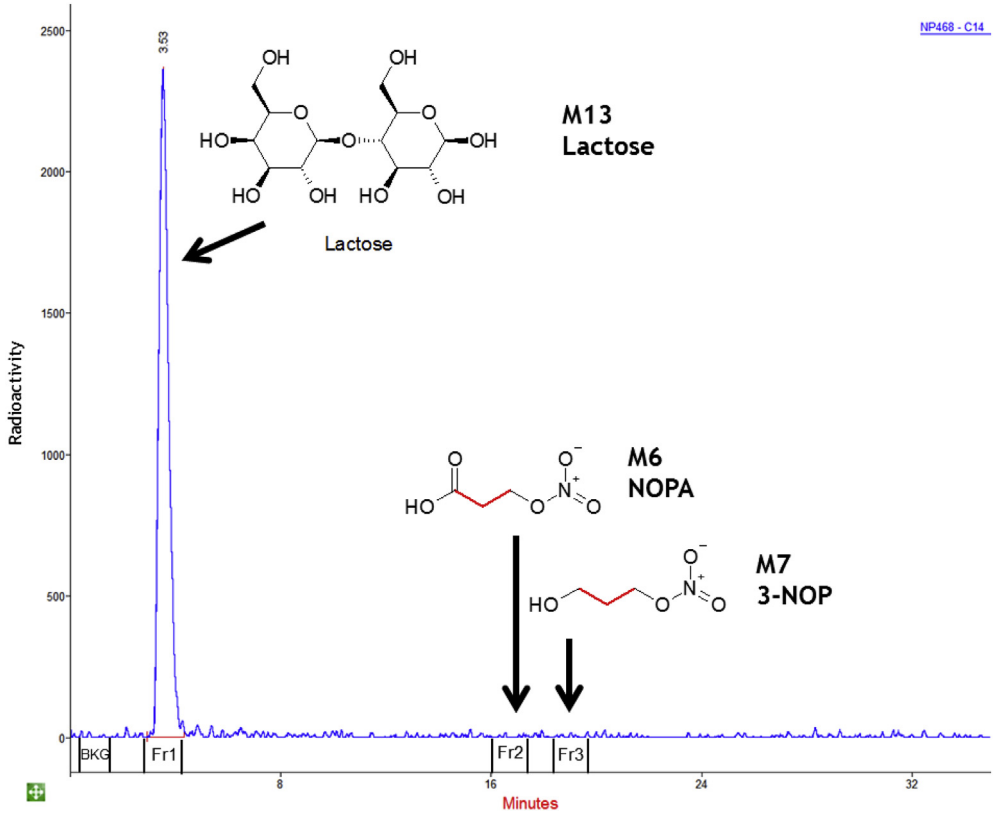

Fig. 6. Radio-HPLC chromatogram of aqueous milk phase on HPLC system S2 (Study 4, Goat 1).

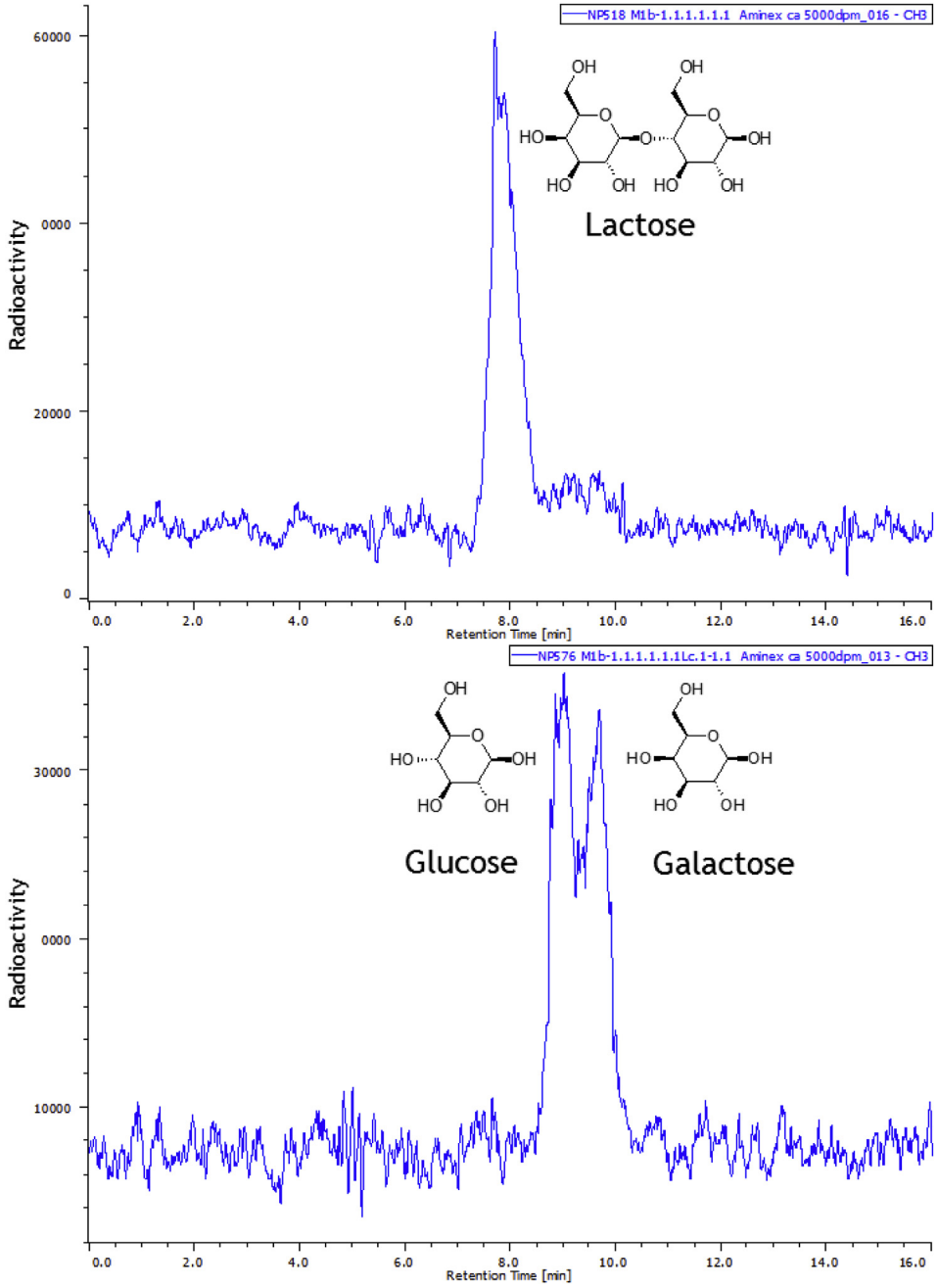

Fig. 7. Radio-HPLC chromatogram (System S6) of the major milk metabolite M13 (Fr1, Fig. 6) before (upper panel) and after (lower panel) treatment with lactase.

or carbohydrates (gluconeogenesis).

Indeed, ruminants use propanoyl-CoA as their major source for gluconeogenesis (Aschenbach et al., 2010) and consequently for synthesis of lactose which was found as the sole radiolabelled substance in the aqueous fraction of milk from a lactating goat treated with ^{14}C-3-NOP. It is not to be expected that either 3-NOP or NOPA would be present in the fat or protein fraction because of their high water solubility (367 g/l and 1000 g/l, respectively). It is likely that the radioactivity associated with protein and fat of edible tissue represents ^{14}C-labelled carbon incorporated into amino acids or fatty acids. Due to the low radioactivity present in edible tissues the hypothesized incorporation of the radiolabel into amino acids/or fatty acids was not pursued. The small molecule nitro-alkanes or nitro-alkanols were metabolized as well into endogenous compounds such as amino acids or acetyl CoA (Zhang et al., 2018; Smith and Anderson, 2013) which is supporting our hypothesis.

Overall, the data indicate that 3-NOP is efficiently and rapidly metabolized via NOPA into endogenous building blocks i.e. endogenous (natural) substances in target animals which predominantly are oxidized to CO_2. Thus, there is equivalence of the metabolism in the target animal (i.e. ruminant) and the rat.

Residues in edible tissues and milk upon 3-NOP administration may be present in minute amounts but unlikely because (i) the majority of the applied radiolabel is exhaled as CO_2, (ii) 3-NOP is quickly transformed into endogenous HPA and further degradation products and incorporated into carbohydrates (i.e. lactose), (iii) 3-NOP and NOPA (the two xenobiotic substances found) have high water solubility and are unlikely to be accumulated in tissues, and (iv) the LOQ of 3-NOP and NOPA in the aqueous phase of the milk was low (200 ppb). However, the compounds were not detected. Therefore, the selection of a marker residue is challenging.

The concentration of radioactivity in the edible tissues of the

Table 2
Distribution of rat plasma metabolites (Study 3) at different time points. Each value represents data from a single animal.

Sampling time point	% of plasma radioactivity			Concentration [nmol/g]		
	1 h	2 h	3 h	1 h	2 h	3 h
Total	**100.0**	**100.0**	**100.0**	**5278**	**3861**	**1152**
Non-extractable	**19.5**	**20.2**	**46.3**	**1029**	**780**	**533**
Extractable	**80.5**	**79.8**	**53.7**	**4248**	**3081**	**619**
- Fraction M1 (highly polar unretained metabolites)	1.9	3.8	14.5	101	148	167
- Fraction M2	2.3	2.0	2.8	122	78	32
- Fraction M3 (HPA)	25.3	48.6	28.3	1334	1877	327
- Fraction M4	0.0	2.3	2.8	0	89	32
- Fraction M5	1.2	0.0	0.0	61	0	0
- Fraction M6 (NOPA)	48.9	23.0	5.3	2581	889	61
- Fraction M7 (3-NOP)	1.0	0.0	0.0	51	0	0

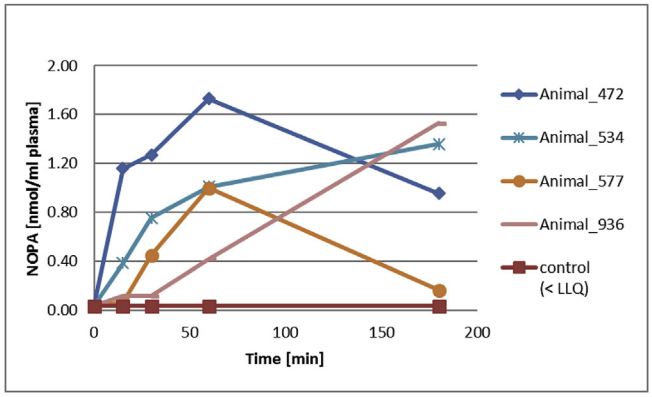

Fig. 8. NOPA plasma kinetics in beef cattle treated with 2 g 3-NOP/day mixed into the feed (approximately 3 mg/kg bw).

lactating goat was low: only approximately 5% of the dose were found in the edible tissues and approximately 6% of the dose were found in milk (see Table 1). In milk most of the radioactivity was associated with lactose. Neither ^{14}C-3-NOP nor ^{14}C-NOPA were found in milk (LOQ 200 ppb) or in rat liver (LOQ 1000 ppb). HPA, lactose and inorganic nitrate are endogenous substances which disqualifies them as marker residue due to natural background levels (EFSA, 2017, VICH GL 46, 2011).

The intake assessment for the consumer is challenging but seems possible using the total radioactive residues (TRR) in the edible tissue thereby discounting identified endogenous substances i.e. radioactivity identified as lactose in milk as well as radioactivity thought to be incorporated into protein or fat. Such an approach would be very conservative and would represent a considerable overestimation of intake.

5. Conclusion

3-NOP is a small molecule with dual chemical functional groups: It is a primary alcohol and an organic nitrate ester. 3-NOP is efficiently metabolized to natural substances being mainly carbon dioxide but also carbohydrates. The proposed metabolism pathway is via oxidation of the alcohol function and hydrolysis of the nitrate ester yielding 3-hydroxypropionic acid (HPA) and inorganic nitrate. HPA is further used by mammalian cells as substrate for synthesis of propanoyl-CoA or acetyl-CoA (thereby liberating carbon dioxide). These metabolic reactions eventually result in the incorporation of 3-NOP carbon into carbohydrates and most likely also into amino acids and fatty acids. This metabolic behavior makes the compound unique and requires adaptation of the standard procedures for intake assessment. Data generated by this series of studies supports the notion that 3-NOP could be safely used to mitigate methane production in ruminant animals.

Conflict of interest and role of funding source

The authors declare no conflict of interest other than employment. AT and RR designed the research, AT and PB supervised the research, AT, RR, PM, and HY wrote the paper, RR served as study director, PM and HY performed the structural elucidation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.02.002.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2019.02.002.

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