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Increased hepatic skatole exposure via rumen infusion increases skatole concentration in peripheral circulation and inter-muscular fat in sheep

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ABSTRACT

Skatole meat taint is unique to pastoral systems where high forage digestibility results in tryptophan-derived skatole production at rates that exceed the clearance capacity of the liver. We hypothesise that under conditions of minimal endogenous skatole production (diet low in rumen degradable protein) a continuous rumen infusion of skatole will elevate hepatic exposure and modify tissue skatole metabolism. A 72 hour rumen infusion of skatole (140 mg/h; n = 6) dissolved in propane-1,2-diol (PD) or PD media alone (n = 6) was performed. Samples of rumen fluid and blood were collected at 0, 2, 4, 6, 8, 12, 16, 24, 48 and 72 hours for determination of skatole concentration. Infusion of skatole increased (P < 0.01) levels of skatole in rumen fluid (21 µg/g), peripheral plasma (154 ng/ml) and inter-muscular fat (4240 ng/g). A two-compartment model was fitted to the rumen and plasma skatole concentration of treated sheep to estimate the rate of transfer from rumen to peripheral plasma ($k = 0.23 \pm 0.03$/h) and the rate of elimination from peripheral circulation ($k = 2.10 \pm 0.56$/h). A negative correlation (P < 0.05) between the rate of elimination and level of skatole deposition in inter-muscular fat was also found.

Keywords: skatole; rumen; hepatic, metabolism.

INTRODUCTION

Undesirable meat flavours arise from ruminant production systems utilising pastures with high rumen degradable protein (RDP) content. These flavours constrain sheep meat marketability and returns, especially in Asian and European markets (Prescott *et al.*, 2001). The volatile indolic compound skatole, produced in the rumen (Carlson *et al.*, 1968), contributes to these undesirable meat flavours (Priolo *et al.,* 2001; Young *et al.,* 2003). Strategies to reduce skatole deposition in meat must also reduce its appearance in peripheral circulation as skatole is rapidly deposited in body fat (Whittington *et al.*, 2004).

The major route of skatole disappearance from the rumen is via absorption into the portal blood (Hammond *et al.*, 1983; Roy *et al.*, 2004) with almost all absorption from the liquid phase occurring prior to the duodenum (Hammond *et al.*, 1984). Skatole in the portal blood flows to the liver where its metabolism could potentially modify the concentration of skatole appearing in the peripheral circulation, thus affecting the deposition of skatole in body fat (Zamaratskaia *et al.*, 2006).

The present study was undertaken as part of a larger programme that aims to discover genes involved in the metabolism of skatole in the ovine liver. It is hypothesised that a 72 hour administration of exogenous skatole in the rumen will modify hepatic skatole metabolism in sheep. The objective of this experiment was to collect hepatic tissue suitable for micro-array experimentation following this contrasting skatole exposure. Additionally the administration of exogenous skatole has enabled the modelling of skatole flux and provided an insight into the variation in skatole metabolism between individual sheep.

MATERIALS AND METHODS

Animals, surgery and feeding

Twelve castrate Suffolk x Romney sheep of 10 months of age and from a single sire were selected following screening for normal liver function using clinical laboratory tests of gamma-glutamyl transferase, glutamate dehydrogenase, total protein, albumin and globulin (NZ Veterinary Pathology). All sheep were surgically fitted with permanent rumen cannula under general anaesthesia. After surgery, the animals grazed a perennial ryegrass-white clover pasture for two months prior to the commencement of the experiment. In order to minimise endogenous skatole production a concentrate diet with low RDP content (13.4 MJ/kg ME; 130 g/kg DM CP; 40 g/kg DM estimated RDP) was designed to replicate the skatole reducing effect of maize feeding shown by Young *et al.* (2003). Animals were adjusted to

the experimental diet during a 14 day period. Sheep were moved indoors for a further 14 day period to adapt to individual metabolism crates and hourly feeding (50 gDM/h), prior to commencement of treatments. Water was available *ad libitum*. All procedures described herein were approved by the Animal Ethics Committee of AgResearch Limited (number 44/03).

Treatments, infusion and sampling

A completely randomised block design (6 sheep per block) was used with period of infusion (n = 2) as a blocking factor. A continuous infusion of propane-1,2-diol (PD; 99.5%; Asia Pacific Specialty Chemicals Limited.) was administered to all sheep at a rate of 5 ml/h via a multi channel variable speed peristaltic pump (Watson-Marlow Ltd.) for 72 hours to allow equilibration of PD concentration in the rumen. Control sheep (n = 6) continued to receive skatole-free PD infusion while treated sheep (n = 6) received PD containing 28 mg/ml skatole (3-methylindole, 98%; Aldrich Chemical Company, Inc.), supplying a total skatole dose of 140 mg/h for a period of 72 hours. Skatole infusate was prepared daily to minimise skatole oxidation prior to administration.

Rumen fluid and peripheral blood samples were collected at 0, 2, 4, 6, 8, 12, 16, 24, 48 and 72 hours relative to the start of the treatment infusion. Whole rumen digesta samples were strained through muslin cloth to allow collection of the liquid fraction. Aliquots of fluid were snap-frozen in liquid nitrogen to inhibit microbial skatole production and stored at -20°C. Peripheral blood was collected via a jugular catheter into syringes containing potassium-EDTA anticoagulant. Blood samples were kept chilled, plasma was separated by centrifugation, snap-frozen in liquid nitrogen and stored at -85°C. All sheep were euthanased at 72 hours from the start of their treatment infusion period. Samples of liver and inter-muscular fat from the hind leg were collected, snap-frozen in liquid nitrogen and stored at -85°C.

Analytical methods

Skatole concentration in rumen fluid was determined using the high performance liquid chromatography (HPLC) method of Mattivi *et al.* (1999) modified by Tavendale *et al.* (2005). Plasma skatole concentration was determined using the ether extraction method of Claus *et al.* (1993) and HPLC analysis (Schreurs *et al.*, 2003). The skatole content of inter-muscular fat was determined using gas chromatograph mass spectrometry following simultaneous distillation-extraction as described by Schreurs *et al.* (2006).

Modelling skatole flux

A two-compartment model (Figure 1) was fitted to the rumen and plasma concentration data (Thomas & Beadle, 1985) from six skatole-infused sheep using the population study algorithm from WinSAAM (Wastney *et al.*, 1999).

FIGURE 1: A two-compartment model fitted to the rumen (compartment 1) and plasma (compartment 2) skatole data of rumen skatole-infused sheep provides an estimated rate of transfer from rumen to peripheral blood $k(2,1)$ and an estimated rate of clearance from the peripheral circulation $k(0,2)$.
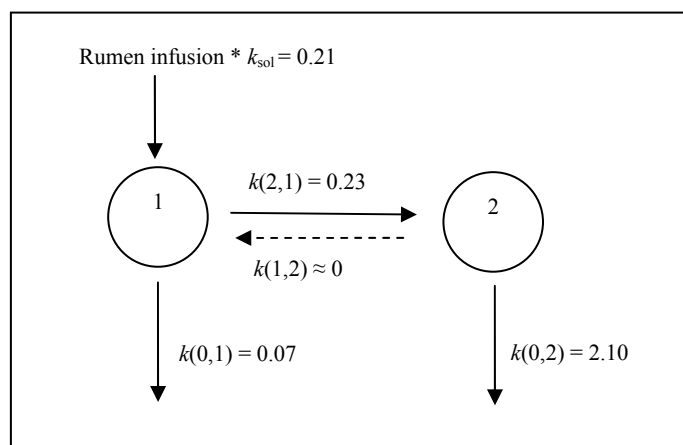
Compartment 1 was assumed to be the rumen liquid phase. Skatole entering into this compartment was calculated as the hourly rate of skatole infusion multiplied by an assumed partition coefficient of skatole into the liquid phase (k_{sol}) of 21% (Hammond *et al.*, 1984), with the remaining skatole precipitating and becoming associated with the particulate fraction. The volume of compartment 1 was assumed to be 10% of the liveweight of the animal (de Vega *et al.*, 1998). Compartment 2 represents absorbed skatole, assuming an apparent volume of distribution of skatole of 1.3 l/kg LW (Friis, 1993).

First order kinetics were assumed for the two compartments, with rates of transfer defined as $k(2,1)$ for transfers from rumen to plasma and $k(1,2)$ for transfer from plasma to rumen. Initial fit indicated that $k(1,2)$ was close to zero and was therefore removed from the model (Figure 1). Additional loss from the rumen was assumed to occur in the form of passage of liquid to the post-ruminal compartment: $k(0,1) = 0.07$ (average of Karsli & Russell, 1999 and Uden *et al.*, 1982).

The rate of disappearance from compartment 2 (i.e. $k(0,2)$) was assumed to represent the capacity of individual sheep to metabolise skatole and skatole accretion into body tissues. Skatole concentrations measured from

control animals (multiplied by rumen liquid and plasma volume) were used to define the initial conditions for the two compartments.

Statistical analysis

Rumen and plasma data was statistically analysed using GenStat, version 8.11 (Lawes Agricultural Trust, 2005). As variance of the measurements in the control group was considerably lower than those of the skatole group, a non-parametric Kruskal-Wallis analysis of variance test was used to compare the skatole concentration data in rumen fluid and plasma between the two groups. Tests of rumen and plasma data were done at 6, 16 and 72 hours. Skatole concentration in inter-muscular fat was analysed using the MIXED procedure of SAS (2002, v. 9.1 SAS Institute). Fixed effects included treatment, while heterogeneous variances between groups were dealt with by using the grouping variance option in the "repeated" statement of the procedure, using sheep as the subject (i.e. random effect). A simple covariance matrix (i.e. variance components) was deemed to be the most appropriate structure underlying the data. Significant difference between treatments was declared when the probability was less than 0.05.
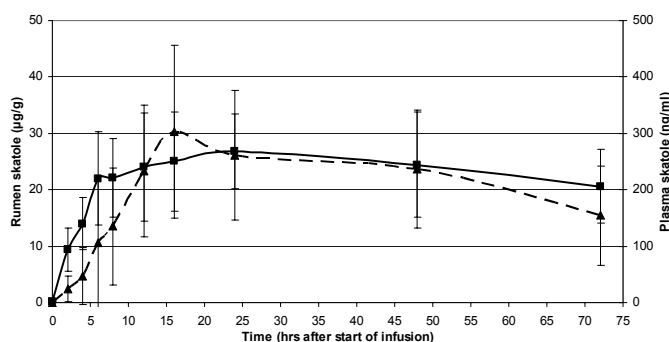
RESULTS

Animals, intakes and infusion rates

There was no significant difference between the mean live weight of sheep allocated to skatole (40.5 kg) and control (42.1 kg) infusions (P = 0.44). All sheep ate 100% of the pelleted ration offered hourly during the infusion period. Actual rate of skatole infusion was 140.84 mg/h.

Rumen, plasma and fat skatole

The endogenous production of skatole (i.e. from rumen degradation of dietary tryptophan) was effectively minimised by the diet. Mean skatole concentration (±SEM) was 0.02 ± 0.02 μg/g in rumen fluid and 0.74 ± 0.77 ng/ml in plasma of control sheep during the treatment period. There was no significant effect (P = 0.90) of period of infusion (blocks) on rumen or plasma skatole concentrations measured. Infusion of skatole generated a rapid rise in skatole concentration in both the liquid fraction of the rumen and peripheral blood (Figure 2). Mean skatole concentration in the rumen fluid and peripheral circulation of skatole-infused sheep was greater (P < 0.01) than control sheep at all times following the start of infusion. At 72 hours, the mean concentration of skatole (± SEM) in rumen fluid and plasma was 20.6 ± 6.5 μg/g and 154.2 ± 87.3 ng/ml in treated sheep and

0.01 ± 0.03 μg/g and 0.89 ± 0.91 ng/ml in untreated sheep respectively.

FIGURE 2: Mean skatole concentration in rumen fluid (■; μg/g) and plasma (▲; ng/ml) of sheep receiving a continuous rumen infusion of skatole (141 mg/h; n = 6). Skatole concentrations in rumen fluid (0.02 ± 0.02 μg/g) and plasma (0.74 ± 0.77 ng/ml) of control sheep (n = 6) were unchanged throughout the treatment period (data not shown). Error bars shown are the SEM.

Skatole concentration in inter-muscular fat from the hind leg was (P < 0.001) increased by the skatole infusion resulting in a mean (± SEM) concentration of 4240 ± 1720 ng/g and 15 ± 15 ng/g in skatole-infused and control groups, respectively. Fitting the model described in Figure 1 to the experimental data resulted in estimates for parameter $k(2,1)$ of 0.22 ± 0.02/h and for parameter $k(0,2)$ of 2.08 ± 0.35/h via all routes of clearance. Rates of skatole clearance in treated sheep ranged from 1.11/h to 4.47/h of the total absorbed skatole pool.

TABLE 1: Mean skatole flux in treated sheep (n = 6) as predicted using the two compartment model described in Figure 1, using population parameters generated from the WinSAAM population algorithm.

Parameter	Rate (g/day)	90% confidence interval [1]
Inflow into rumen liquid	0.710	Not applicable
Outflow from rumen to intestine $k(0,1)$	0.160	0.157 – 0.164
Absorption from rumen $k(2,1)$	0.549	0.545 – 0.553
Clearance from circulation $k(0,2)$ [2]	0.549	0.545 – 0.553

[1] Of mean given a two tailed normal distribution.
[2] Assuming transfer from peripheral blood to rumen $k(1,2)$ to be zero.

There was a negative correlation (-0.73, R^2 = 0.47, P < 0.05) between rate of plasma skatole elimination and concentration of skatole in hind leg fat depots.

DISCUSSION

The present experiment has minimised endogenous skatole production and generated a significant elevation in skatole concentration in both rumen fluid and peripheral circulation via a continuous administration of skatole to the rumen.

The high rates of elimination of skatole from the peripheral circulation observed in this study are consistent with the high capacity of skatole metabolism in tissues. According to the model described herein, estimated pool sizes of absorbed skatole ranged from 5 to 18 mg. In pigs, hepatic detoxification of endogenous (hind gut) skatole has been reported to reach up to 3 mg/h (Babol *et al.,* 1998).

Hepatic detoxification of skatole is mediated by cytochrome P450 microsomal enzymes (Diaz *et al.,* 1999) and these enzymes can be induced by skatole in isolated pig hepatocytes (Doran *et al.,* 2002). In this study, induction of P450 enzymes in the liver and increase of clearance of skatole by other tissues (e.g. lung, kidney, fat deposition: Laue *et al.*, 1998) may account for the high rates of clearance of absorbed skatole observed. The negative correlation between estimated plasma skatole clearance and body fat deposition suggests that changes in activity of hepatic enzymes may be partly responsible for the high rates of skatole clearance described by our model. Studies where cytochrome P450 activity in porcine liver is increased report a negative correlation with skatole concentration in body fat (Squires & Lundstrom, 1997; Babol *et al.*, 1998; Zamaratskaia *et al.*, 2006).

Estimated rates of skatole clearance were highly variable between individuals suggesting that significant variation in the hepatic and / or extra-hepatic response to skatole exposure may exist within the New Zealand sheep population.

The generation of contrasting skatole exposure to the liver has enabled the collection of hepatic tissue likely to have differentiated skatole-related gene expression. These tissues may assist in the identification of genes involved in mediating skatole metabolism and whose activity may ultimately explain the variable deposition of skatole in body fat that we have observed between individuals.

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