Endogenic Nortestosterone in Cattle?*

Hubert F. de Brabander, Johan van Hende and Peter Batjoens
Department of Veterinary Food Inspection, lab Chemical Analysis, RUG, Salisbury Laan, 133 B-9820 Merelbeke, Belgium

Lutgart Hendriks and Jef Raus
Dr. L. Willems-Institute, Universitaire Campus, B-3590 Diepenbeek, Belgium

Felix Smets and Gaspard Pottie
IHE, J Wytsmansstraat, 14 B-1050 Brussels, Belgium

Leen van Ginkel and Rainer W. Stephany
RIVM, A Van Leeuwenhoeklaan, 9 NL-3720 BA Bilthoven, The Netherlands

When residues of nortestosterone (NT) were found in the urine of cattle, racehorses or bodybuilders, exogenic administration was thought to be proven. In previous literature, no records were found of the endogenic presence of this molecule. In the horse-racing world, Houghton and Courthot found that NT is normally present in the urine of the stallion. Belgian and Dutch researchers found that NT is also present in the urine and edible parts of the intact boar. Vandenbroeck *et al.* (1991) suggested the endogenous presence of NT (in the β form) in the pregnant cow. Meyer (1992) reported the presence of NT (in the α form) in relatively high amounts in the urine of the cow *peri-partum* and the neo-natal calf. These observations may have important consequences for veterinary meat inspection in the EU. Therefore, in Belgium a large scale experiment was set up in co-operation with the EU Community Reference Laboratory (RIVM). In this paper the present state of the results in this area is presented. A large number of urine samples (>50) of pregnant non-treated cows were collected and analysed by gas chromatography–mass spectrometry (GC–MS) in 4 different laboratories. Further samples (>100) were taken, but only analysed in one laboratory. The results proved clearly that NT may indeed be detectable in the α form in the urine of pregnant cows, from at least 2 months, but most probably from 4–5 months before *partus*.

Keywords: Gas chromatography–mass spectrometry; nortestosterone; hormonal residue; cattle; endogenic hormone; metabolite; interlaboratory test; steroid

Introduction

Laurabolin, decabolin and durabolin, which all contain nortestosterone fatty acid esters, were circulating on legal and illegal markets and were used in cattle fattening, horse racing and by athletes. When residues of nortestosterone (NT) (nandrolone) were found in the urine of cattle, racehorses or bodybuilders, exogenous administration was thought to be proven. In previous literature, no records were found of the endogenic presence of this molecule. Tuinstra *et al.*[1] described 17α-nortestosterone (17αNT) and estranediols as the most important metabolites of nortestosterone in cattle (Fig. 1). These products were only detected in animals treated with nortestosterone or nortestosterone derivatives (typically esters).

In the horse-racing world, Houghton[2] and Courthot[3] found that NT is normally present in the urine of the stallion. Belgian and Dutch researchers found that 17βNT is present in the urine and edible parts of the intact boar. These observations were initially presented to working groups, such as the 'Veterinary Inspection working group' and the 'BENELUX working group', and later published by Maghuin-Rogister,[4] Van Ginkel *et al.*[5] and Debruyckere *et al.*[6] Rizzo *et al.*[7] later reported the endogenic origin of nortestosterone by analysis of boar testis. Since then, for meat inspection purposes, boars were no longer sampled for nortestosterone analysis. As another consequence pig meat (and pig-meat products) may no longer be controlled on NT in trade between EU countries.

The first suspicion that NT could also be endogenic in cattle was based on an analytical artefact[8] (confusion between 17α-testosterone and 17αNT) in the urine of male veal calves. This problem did not exist in laboratories that used high-performance liquid chromatography (HPLC) purification prior to high-performance thin layer chromatography (HPTLC). Meyer *et al.*[9] reported in 1988 that NT could be present in untreated veal calves by consumption of contaminated milk replacers. Vandenbroeck *et al.*[10] suggested, for the first time, the endogenic presence of NT (βNT, but not the α form) in the urine of the pregnant cow.

However, this could not be confirmed by studies at other laboratories. The Dr. L. Willems-Institute (R1) and the RUG, Department of Veterinary Food Inspection, lab

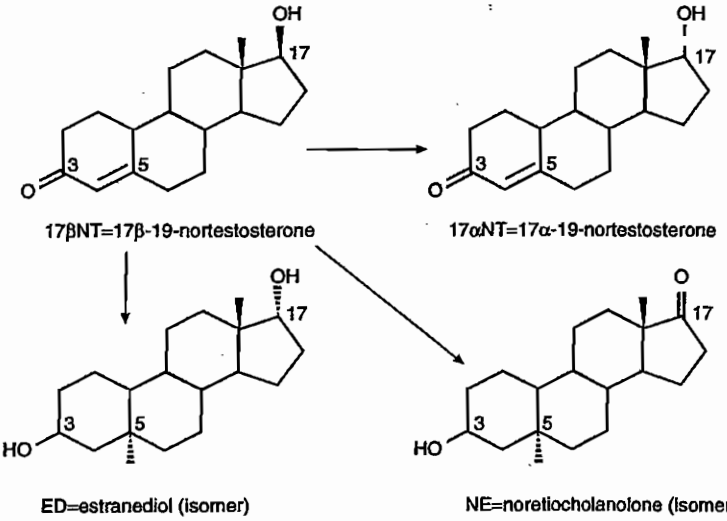

Fig. 1 Metabolic pathways of nortestosterone.

* Presented at the Second International Symposium on Hormone and Veterinary Drug Residue Analysis, Oud St-Jan, Bruges, Belgium, May 31–June 3, 1994.

Chemical Analysis (D1) carried out a limited number of scientific experiments on pregnant cattle, following the publication of work by Vandenbroeck *et al.*[10]

The results are summarized below. More details of these studies may be found in the thesis of Van Den Braembussche.[11]

During regulatory control, 25 female animals were sampled (2 were not pregnant and 23 were in different stages of pregnancy). Eighteen urine samples, taken during the last 4 months of pregnancy, were analysed by (RIA) at R1. The stage of pregnancy was estimated. NT–RIA responses were found, as described by Vandenbroeck *et al*,[10] and within the same range (2–10 ppb). The highest RIA responses were found during the last 2 months of pregnancy.

Table 1 and the results of Vandenbroeck clearly proved that during the last months of pregnancy there is a rise in the response to the NT antibody used (NT antibody obtained from lab d'Hormonology, Marloie, Belgium). However, the presence of NT in the urine samples could not be confirmed by HPLC–HPTLC and high-performance liquid chromatography–gas chromatography–mass spectrometry (HPLC–GC–MS). With these methods, neither 17βNT, nor 17αNT could be detected in the 25 samples by either laboratories. Possibly, the NT-RIA response could be due to other products (*e.g.*, steroids) excreted during this phase of pregnancy.

However, very high concentrations of α-estradiol were detected in the urine of animals in the last stage of pregnancy. It was found that these high concentrations of α-estradiol could generate false positive βNT results using selected-ion monitoring (SIM) without using a full-scan chromatogram to trace interfering substances (Leyssens *et al.*).[12] The principle of these analytical pitfalls in SIM was worked out further, as a theoretical example, by De Brabander *et al.*[13] For the 3 laboratories involved in these experiments (D1, R1 and IHE), the detection of βNT in the urine of pregnant cattle was considered as an analytical artefact caused by isotope interference.

During the International Symposium on Hormone and Veterinary Drug Residue Analysis (Ghent, Belgium, 1992), Meyer[14] reported the presence of NT (in the 17αNT form) in relatively high amounts in the urine of the cow *peri-partum* and the neo-natal calf. As these observations were in contradiction to the results of our limited experiment, and may have important consequences for veterinary inspection in the EU, it was decided a larger scale experiment would be set up in co-operation with the EU Community Reference Laboratory (RIVM).

Experimental

The methods used here have been described and validated previously.[15–17] A short summary of the methods used follows.

The samples were analysed by using HPLC–GC–MS[15] at IHE and D1. Urine (25 ml) was hydrolysed with *Helix pomatia* juice IBF, Clichy, France and extracted with ether. The crude extract was cleaned up with HPLC with fraction collection.[15] The fractions were derivatized to trimethyl silyl (TMS) enol-TMS ether derivatives with (*N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA)–trimethylsilyl iodide (TMSI)–dithiothreitol (DTE) and analysed with full-scan MS on a Finnigan MAT (San Jose, CA, USA) Magnum

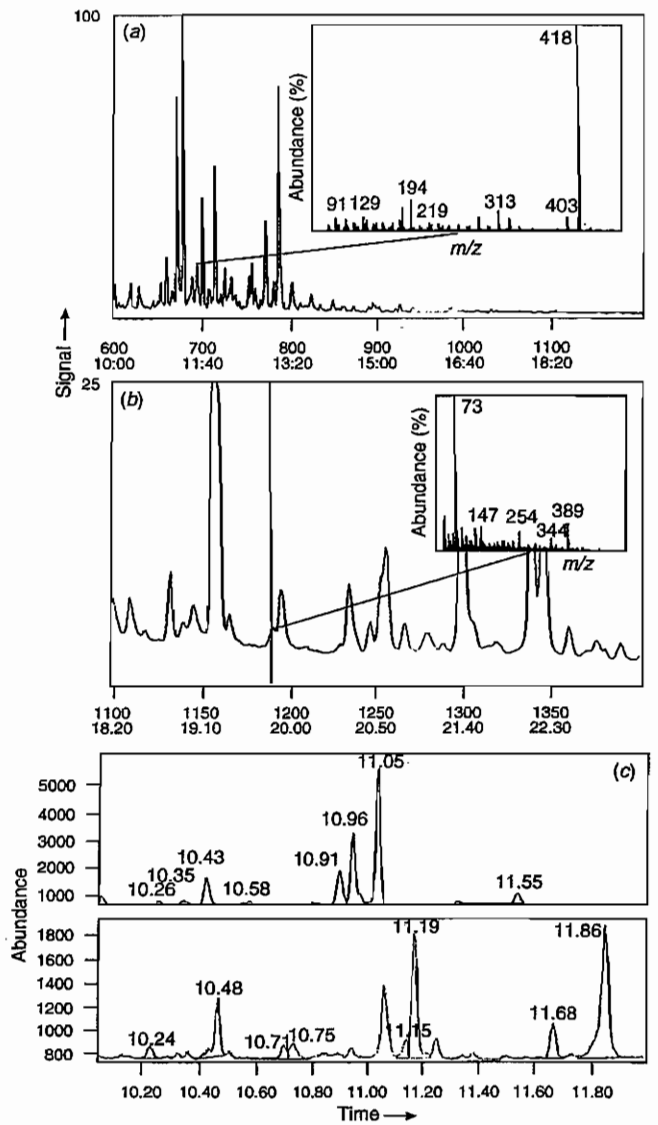

Fig. 2 Examples of chromatograms and mass spectra obtained with 3 different methods. *a*, IHE and D1: chromatogram and full-scan mass spectrum of NT-di TMS in a urine sample; *b*, R1: chromatogram and full-scan mass spectrum of NT-EO-1 TMS-1 in a urine sample; *c*, RIVM: quantitative determination of 17αNT-di HFB in a urine sample. 17αNT, $m/z = 666$; $t = 10.91$, internal standard NT-d3: m/z 669, $t = 11.19$.

Table 1 RIA responses of NT in the urine of pregnant cattle (ppb NT equivalents)*

Pregnancy status*	Sample no.	NT (mean)	Individual NT values
$P-120$	6	0.7	0.1, 0.2, 0.4, 0.7, 1.2, 1.5
$P-90$	1	2.4	2.4
$P-60$	3	4.9	1.3, 3.1, 10.2
$P-30$	8	3.9	1.0, 2.4, 3.1, 3.8, 4.2, 4.7, 5.4, 6.3

* *partus* = 0 (taken as zero point).

Table 2 Some pregnancy data of cattle (Belgian breeds)

Breed	Mean*	s_r†	No. of animals
White-blue	282	6	2344
Red pied	280	5, 8	27197
Red	283	5, 8	6376
Black pied	280	5, 5	83185
White-red	282	5, 6	123235

* Days of pregnancy (mean).
† Standard deviation (pregnancy days).

(ITS40) system. The column used was a fused silica HP-Ultra 2 of 25 m × 0.2 mm i.d. with 0.25 μm film thickness (Hewlett-Packard, Palo Alto, CA, USA). For quantification, an internal standard ((16,16,17β-$^2H_3$)nortestosterone (NTd_3) was used. The detection limit of this method was 0.5 μg l^{-1}.

At R1, a combination of solid phase extraction on 3 columns (^{18}C, silicagel and amino column) and HPLC purification was used as clean-up on 50 ml of urine samples.[16] The samples were enzymically deconjugated (*Helix pomatia*) after the first column. GC–MS on a part of the pooled HPLC fractions was performed with ethoxime-trimethylsilyl derivatives (EO-TMS). The GC–MS was a Varian Saturn I system (Varian, Walnut Creek, CA, USA). The column used was a fused silica DB-5 MS, 30 m × 0.32 mm i.d. with 0.25 μm film thickness. For quantification, equilenin (*d*-1,3,5(10),6,8-estrapentaen-3-ol-17-one) and androsterone (5α-androstan-3α-ol-17-one) were used as internal standards. The detection limit of this method was 0.5 μg l^{-1}.

In the RIVM,[17] urine (5 ml) was extracted with an automated (automated sample preparation, extraction and concentration, ASPEC) procedure on a C_{18} and an amino-solid-phase extraction (SPE) cartridge. Prior to extraction, the internal standard NT*d*3 was added and the samples were enzymatically deconjugated (*Helix pomatia*). The primary extracts obtained after SPE procedure are further cleaned up by immuno affinity chromatography (IAC). After removal of the solvent, the dry residue is derivatized with heptafluorbutyric acid anhydride (HFBA) and the diHFB-NT derivatives are determined with GC–MS (HP5890A gas chromatograph with a fused silica DB1 column of 30 m × 0.25 mm i.d. with 0.25 μm film thickness and HP-5989A MS engine). The limit of detection was 0.2 μg l^{-1}, but a limit of decision of 0.5 μg l^{-1} was used in this study.

In Fig. 2 some examples of the chromatograms and mass spectra obtained with the 3 different methods are given.

Table 3 Results of analyses on α-nortestosterone (ppb, 17αNT)

Pregnancy stage *versus partus*	Animal	Lab. 1	Lab. 2	Lab. 3	Lab. 4
P − 21	‡5455	Pos	2.3	4.6	—
P − 18	8007	Pos	—	5.4	5.6
P − 9	†8315	Pos	1.4	3.9	0.8
P − 6	*8890	Pos	2.4	5.8	3.8
P − 4	1960	Pos	3.8	5.0	5.1
P − 4	9805	Pos	2.8	6.5	4.8
P − 1	1585	Pos	4.7	7.2	3.3
P = 0	*8890	Pos	4.0	6.3	2.2
P = 0	*8890	Pos	1.5	4.3	0.7
P = 0	8515	Pos	4.0	5.4	3.2
P = 0	‡5455	—	—	4.6	9.3
P = 0	‡5455	—	Neg	Neg	Neg
P + 1	*8890	Neg	Neg	Neg	0.9
P + 1	†8315	Neg	Neg	Neg	—
P + 1	†8315	Neg	Neg	Neg	Neg
P + 1	‡5455	Neg	Neg	Neg	0.6
P + 2	5805	Neg	0.7	0.6	Neg
P + 2	*8890	Neg	Neg	Neg	—
P + 3	*8890	Neg	Neg	Neg	Neg
P + 3	†8315	Neg	Neg	Neg	Neg
P + 3	†8315	Neg	—	Neg	Neg
P + 4	*8890	Neg	Neg	Neg	Neg
P + 5	1960	Neg	Neg	Neg	Neg
P + 5	9805	Neg	Neg	Neg	Neg
P + 6	*8890	Neg	Neg	—	Neg
P + 6	*8890	Neg	Neg	Neg	Neg
P + 5	1585	Neg	Neg	Neg	Neg

P = 0, samples taken at different hours; P − *x*, *x* days before *partus*; P + *x*, *x* days after *partus*. Neg recorded when NT ≤0.5.

Results and Discussion

Urine sampling

One of the problems encountered during this project was obtaining large numbers of urine samples in sufficient quantities of non-treated pregnant animals. Obviously, these samples could not be obtained from routine sampling. Moreover, the stage of pregnancy of the animal sampled was required to be as accurate as possible. We learned that estimation of the stage of pregnancy by visual inspection only (*occulus veterinarius*) is very difficult and the absence of the exact knowledge of the stage of pregnancy in the preliminary experiments was considered as a weak point. The normal duration of pregnancy in cattle is 285 days but in practice, random variations may occur and there are variations between breeds. In Table 2 some data for Belgian breeds of pregnant cattle are summarized. In this investigation the reference point for pregnancy was put on the day of the *partus* ($P = 0$) which could be measured accurately. All further data were expressed as a function of this zero point.

Experiments checking Meyer's observations

The first 40 samples, in our large scale experiment, were taken on a dairy-cow farm, with a fairly good warranty that no nortestosterone abuse had occurred (abuse has no benefit for this type of farming). The animals were normally grazing on the pasture and fertilization took place by natural mating. From up to 2–3 weeks before *partus* (rough estimation by the farmer) the animals were brought into the stables and could be sampled more easily. The date of *partus* was estimated and a urine sample was taken regularly from day, $P - 20$ (*i.e.*, 20 days before *partus*). These samples were frozen at −18 °C and analysed after knowledge of the exact date of *partus*. After *partus*, 3 more urine samples were taken in the period of up to 8 days. Unfortunately, some animals were sold during the experiment and only a rough estimation of the *partus* day was obtained. These samples are not included in Table 3, but only used as additional information. The samples were analysed in one of the laboratories (D1) in order to pre-select samples of

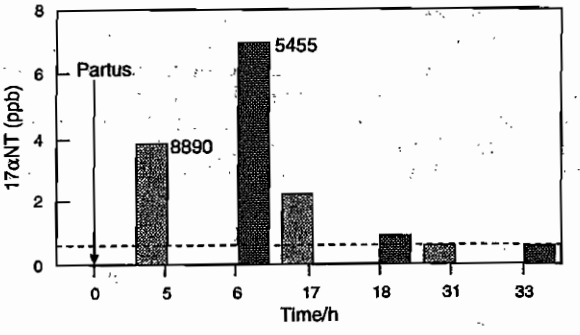

Fig. 3 NT values of urine samples taken shortly after the *partus* (animals 8890 and 5455).

Table 4 NT values found by Meyer [αNT (ppb) determined by HPLC IA]. F = Female calf and M = male calf.

Day	Cow 1 [F]	2 (F)	3 (M)	4 (M)	5 (M)
$P - 18$	—	29	—	—	—
$P - 12$	27	25	—	18	—
$p - 7$	35	20	28	21	—
$P - 1$	—	—	25	20	—
$P + 1$	4	1	18	—	11
$P + 11$	2	1	3	4	1

interest. The samples were then distributed over the 3 other laboratories and analysed quantitatively for NT without pre-knowledge of the stage of pregnancy of the animal.

The results are given in Table 3.

From Table 3, it can be seen that all 4 laboratories detected 17αNT in samples taken shortly before *partus* (period of 21 days before *partus*) in a concentration range of 1–10 ppb (only 3 laboratories did a quantitative analysis). In addition to the data given in Table 3, the 5 other samples, with only a rough estimation of the day of *partus* give analogous results: the between laboratory reproducibility of the results is 49% (s_r (%)). The agreement between the range of the results of the 3 laboratories, which produced quantitative data is acceptable, regarding that the within-laboratory reproducibility at the limit of detection is determined as approximately 25% by the EU Community Reference Laboratory (RIVM). After the *partus*, negative (≤0.5 ppb) or very low 17αNT results (<1 ppb) were found. The data of animals 8890 (marked by *) and 8315 (marked by †), taken at different stages of pregnancy are of particular interest. These samples illustrate very well that NT is detected before, and not after, *partus*. The urine samples of animal 8890 (marked by †) and animal 5455 (marked by ‡) taken at different hours shortly after *partus* (Fig. 2), were also of interest.

Fig. 3 clearly shows that the NT concentration falls below 0.5 ppb within 24 h after *partus*. It should be noted that the urine collected 6 h after partus is (or may be) formed before *partus*. So, excretion and, thus, production of NT ceases quickly at *partus*. This should indicate that the presence and the evolutive status of the placenta is very important in the production of nortestosterone.

The form in which nortestosterone is present in the urine is 17αNT as mentioned by Meyer *et al.* None of the laboratories could detect 17βNT in concentrations greater than 0.5 ppb (considered as an acceptable detection limit) in any of the samples, as mentioned by Vandenbroeck *et al.*[10] Qualitatively, our results confirm and complete the results of Meyer *et al.*[14]: 17αNT may be present in the urine of pregnant cattle from about 20 days before *partus* ($P - 20$). This phenomenon is not dependant on the sex of the newborn calf, neither is it influenced by the parity of the cow (the number of calves she has had).

In Table 4, Meyer's data[14] are converted to our units (ppb, *partus* as zero point). From this Table and Table 3, it can be observed that quantitatively significant differences are found. The concentration of NT found by Meyer *et al.*[14] is much higher than that found in our experiments. However, it should be noted that high-performance liquid chromatography immunoassay (HPLC IA) and GC–MS results are compared.

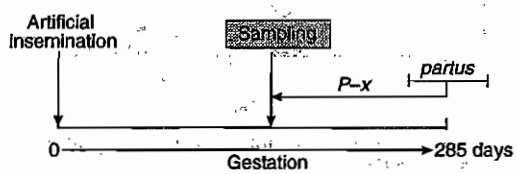

Fig. 4 Estimation of the date of *partus* from artificial insemination data.

Table 5 Preliminary results of the additional experiments

Days before *partus*	No. of analysis	17αNT detected
220–170	2	0
140–100	5	5
100–70	11	8
70–50	25	22
50–40	35	23
29–20	15	12

Possibly, metabolites of NT metabolism cross-react with the HPLC IA method used by Meyer[14] for quantification.

Another important difference between our and Meyer's results[14] is the rate of disappearance of NT in the urine after *partus*. If our hypothesis of the NT-producing placenta, after a certain maturation stage, is right, it should be verified at which time the cow discarded the placenta. Otherwise, we must accept that Meyer[14] with his HPLC IA assay is measuring something more than just 17αNT.

Further experiments

The first question which arose after the confirmation of Meyer's observations, was the length of the period during which 17αNT could be detected in urine. Therefore, the sampling dates were extended forward. However, the further away the sampling from *partus* date, the more difficult an accurate *P-x* can be determined. The strategy using one well known dairy farm, followed in the first experiment, could not be used. Therefore, data from an artificial insemination station were used. On the basis of artificial insemination (AI) data, the date of *partus* of a large number of dairy cows were calculated (Fig. 4). The farms of the animals were located and farmers were requested for sampling permission. Samples were taken and analysed as described previously. Of these samples, the creatinine concentration and density were also determined. The parity of the cow and the sex of the veal calf was noted each time.

In all, 186 urine samples were taken. Only 93 samples have been analysed at some of the laboratories. Samples were divided over the laboratories but up to now, only a limited number of results were available and so the results should be regarded as 'non-confirmed by interlaboratory experiments'. A review of the results is given in Table 5. In the period $P - 140$ to $P - 20$, 17αNT was detected in 70 out of 91 urine samples. In the 2 samples farthest from *partus* ($P - 173$ and $P - 223$) no 17αNT could be detected.

Discussion

From the data, given above it is concluded that 17αNT is present in most of the urine samples taken in the last-half period of pregnancy. So it is confirmed that the steroid is secreted or produced in the pregnant cow. In this context an old habit of Flemish farmers to improve the quality of cow meat by pregnancy (slaughter of the cow after 4 months of pregnancy) could possibly be explained by the anabolizing effect of the steroids generated.

However, it stays an open question why concentrations of 2–9 ppb 17αNT were not detected during the first experiment following the article of Vandenbrouck.[10] It is puzzling why 3 laboratories, with extensive experience in steroid analysis, could not detect 17αNT in the 23 samples taken during regulatory control and later detect 17αNT, without problems, in samples taken at dairy farms. Moreover, in this period, no significant improvement in analytical methods or detection limit has occured. Recently in the slaughterhouse, a veterinary inspector took a urine sample of a cow, which was 7 months pregnant and no 17αNT could be detected. The clinical estimation of the stage of pregnancy, lactation, and the breed of the animals could play a role in the production of 17αNT.

Conclusion

From a large number of samples of pregnant non-treated cows, analysed by GC–MS in 4 different laboratories, it is proved clearly that 17αNT may indeed be present in the urine of pregnant cows from at least 4 months before *partus*. In the samples analysed, 17βNT was not detected in concentrations

greater than 0.5 ppb. The experiment should be continued for other species because in preliminary experiments we also found 17αNT in the urine of pregnant sheep, goats and deer. Furthermore the sampling strategy of the EU has to be reviewed if the presence of endogenous NT in cows urine during gestation is confirmed in a sufficient number in other member states.

References

1 Tuinstra, T., Kienhuis, P., Van Mazij, R. G., and Traag, W., *Proceedings of the International Symposium of Veterinary Laboratory Diagnosticians, Amsterdam, 1986*, pp. 765–767.
2 Houghton, E., Copsy, J., Dumassia, M. C., Haywood, P. E., Moss, M. S., and Teale, P., *Biomed Mass, Spectr*, 1984, **11**, 96.
3 Courtot, D., C.R. Acad Sc Paris 299 III 6 139–141.
4 Maghuin-Rogister, G., Bosseloire, A., Gaspar, P., Dasnois, C., and Pelzer, G., *Ann. Méd. Vet.*, 1988, **132**, 437.
5 Van Ginkel, L. A., Stephany, R. W., Zoontjes, P. W., Van Rossum, H. J., Blitterswijk, H., and Zuijdendorp, J., *Tijdschr diergeneeskunde*, 1989, **114**, 311.
6 Debruyckere, G., Van Peteghem, C., De Brabander, H. F., and Debackere, M., *Vet. Q.*, 1990, **12**, 247.
7 Rizzo, A., Alitupa, E., Berg, S., and Hirvi, T., *Proceedings of Euroresidue II, Veldhoven, 1993*, ed. Haagsma, N., Ruiter, A., and Czedik-Eysenberg, P. B., pp. 581–85.
8 De Ridder, E., Thesis, University of Ghent, Belgium, 1989.
9 Rapp, M., and Meyer, H. D. D., *J. Chromatogr.*, 1989, **489**, 181.
10 Vandenbroeck, M., Van Vyncht, G., Gaspar, P., Dasnois, C., Delahaut, P., Pelzer, G., De Graeve, J., and Maghuin-Rogister, G., *J. Chromatogr.*, 1991, **564**, 405.
11 Vanden Braembussche, C., Ph.D. Thesis, University of Ghent, Belgium, 1990.
12 Leyssens, L., De Brabander, H. F., Pottie, G., Smets, F., and Hendriks, L., Doc Benelux Ec Unie SP/LAB/h 1991, 91-10.
13 De Brabander, H. F., Batjoens, P., Van den Braembussche, C., Dirinck, P., Smets, F., and Pottie, G., *Anal. Chim. Acta*, 1993, **275**, 9.
14 Meyer, H. D., Falckenberg, D., Janowski, T., Rapp, M., Rosel, E., Van Look, L., and Karg, H., *Acta. Endocrinol.*, 1992, **126**, 369.
15 Smets, F., and Vandewalle, G., *Z. Lebensm.-Unters. Forsch.*, 1984, **178**, 38.
16 Leyssens, L., Royakers, E., Gielen, B., Missotten, M., Schoofs, J. Czech, J., Noben, J. P., Hendriks, L., and Raus, J., *J. Chromatogr.*, 1994, **654**, 43.
17 Van Ginkel, L. A., Stephany, R. W., Van Rossum, H. J., Steinbuch, H. M., Zomer, G., Van de Heeft, E., and De Jong, A. P. J. M., *J. Chromatogr.*, 1989, **489**, 111.

Paper 4/03396E
Received June 6, 1994
Accepted September 15, 1994