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Determination of 5-vinyloxazolidine-2-thione residues in ruminant tissues

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INTRODUCTION

In the EEC countries there is a ban on the use of 'anti-hormones' in cattle fattening. In Europe, the word 'anti-hormones' is synonymous with thyreostatic drugs. However, this nomenclature is not correct : 'anti-hormones' counteract the action of a hormone, not its production. Therefore we will use the nomenclature thyreostatic drugs (TS) throughout this article. The weight-gain caused by the administration of TS to cattle consists mainly of an increased filling of the gastro-intestinal tract and augmented water retention in the slaughter animals. Therefore meat from animals, treated with thyreostatic drugs, may be exudative and of inferior quality. The presence of residues of these TS in meat may also constistute a health hazard. In our laboratory intensive research was directed to the detection and the determination of thyreostatics. For the determination of residues of the important group of synthetic thyreostatic drugs, the thiouracils, specific and sensitive methods were described (1)(2) and the excretion and distribution of methylthiouracil (MTU = 4(6)-methyl-2-thiouracil) in cows was studied (3). Next to the synthetic drugs, some products, present in material used as animal fodder, have a thyreostatic effect. The natural goitrogens of most interest are hydrolysis products of glucosinolates, present in plants of the Cruciferae and related families (4)(5). Most of these sulfur compounds act through metabolisation to the thiocyanate ion. The quantitative determination of SCN^- and the excretion and distribution of SCN^- in laboratory animals was studied (6).Oxazolidine-2-thiones, also called goitrines, are a group of cyclic degradation products of glucosinolates which are very potent goitrogens. The best known member of this group, 1-5-vinyloxazolidine-2-thione (5-VTO, goitrin) has 133 % of the goitrogenic activity of propylthiouracil in man (5)

Analytical data on oxazolidine-2-thione concentrations in body fluids and tissues of animals are scarce ,mainly due to the absence of sensitive and low cost determination methods.The transfer of high quantities of thioglucoside derivatives from fodder into milk was first measured using spectrophotometric methods(7)(8). The distribution and excretion of 5-VTO in rat was studied with liquid scintillation counting (^{35}S-VTO) (9). The presence of VTO in blood, urine and faeces of rats, after administration of rapeseed meal to the animals, was detected using a gaschromatographic method (Daxenbichler et al.(10))(11,12). The same method was used by Van Etten et al. to investigate VTO residues in meat of cows, given a fodder regime with rapeseed meal (13). Unfortunately the detection limit of the GC method used was too high (ca 1 ppm) and no residues were detected.

In an earlier investigation (14) we described a more sensitive (detection limit ca 1 ppb), simple cheap and rapid method for the quantitation of oxazolidine-2-thiones in biological fluids. In this paper we report an adaptation of the procedure for the determination of these thyreostatic drugs in tissues (thyroid, meat). Using this technique the concentration and stability of goitrins in body fluids and tissues of ruminants after the administration of the drug or its precursor progoitrine was studied.

EXPERIMENTAL

-Analytical Procedure

The determination of 5-VTO in plasma, urine and milk was carried out using the analytical procedure described previously(14).For the determination of 5-VTO in tissue 2 g tissue (thyroid, liver, kidney or meat) was homogenized with 5 ml distilled water in a glass centrifuge tube using an ultra-turrax. After centrifugation at 2500 rpm (1000 g) 2 ml of the supernatant (equivalent to 0.62 g tissue) was extracted with 5 ml phenylmercuricacetate (PMA) solution as described previously (14)

-Determination of thyroxine (T_4) in bovine serum

The thyroxine concentration in bovine serum was determined using an ELISA method (Enzyme Linked Immuno Sorbent Assay : Boehringer Mannheim (Enzyme Test® T_4)). The results are expressed as µg T_4/100 ml serum.

RESULTS AND DISCUSSION

-Stability of 5-VTO in biological samples

In view of the scarce litterature data on the stability of 5-VTO in milk, urine and plasma this study was initiated. For both raw and cooked milk, the concentration of oxazolidine-2-thiones was found to be stable during storage at freezing conditions over the whole storage period investigated (6 months). Plasma and urine samples of three cows were analyzed after storage in the freezer during 1,3 and 6 months: no decrease of the 5-VTO concentration was found with increase of the storage period.

From these experiments it was concluded that freezing (-25°C) allow a storage of the biological samples (milk, urine, plasma) over prolonged periods without loss of oxazolidine-2-thiones.

-Elimination of 5-VTO after intraruminal administration to a goat

An aqueous 5-VTO solution (360 mg in 50 ml solution) was prepared by hydrolysis of rapeseed meal (50 g) with myrosinase (thioglucoside glucohydrolase E.C. 3.2.3.1). After morning milking this solution was infused intraruminally by way of a stomach tube to a goat Heparinized blood and urine samples were taken each hour during 13 hours post infusion. Milk samples were taken every 2 hours. The samples were analysed and the results were corrected for a mean extraction yield of 86%. The results are presented as a plot of the logarithm of the concentration of 5-VTO (ppm) versus time (Fig.1).

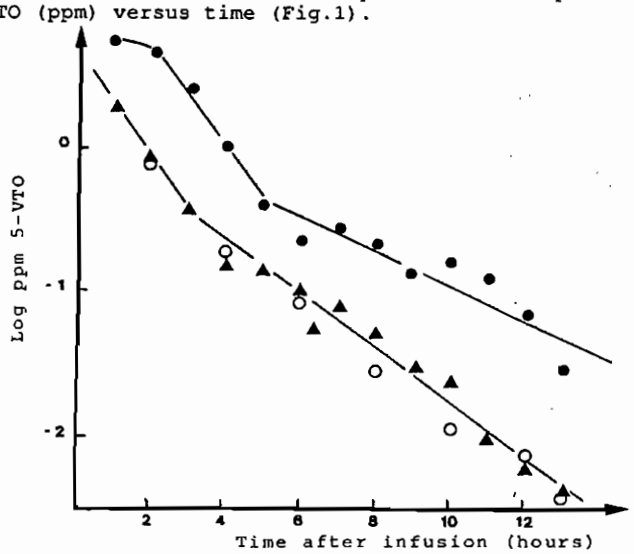

FIG.1 : Elimination of 5-VTO in milk (O),plasma (▲) and urine (●) after administration of 5-VTO

The concentrations of 5-VTO in milk are similar to those in plasma. Compared with plasma, the 5-VTO was concentrated in urine 5-10 fold. The elimination of 5-VTO in plasma or milk and urine versus time can be represented by two straight parallel lines.

The elimination speed of 5-VTO is decreased ca 4-5 h post infusion (nod in the curves). From the second part of the elimination curves the average half-life of 5-VTO in goat after single intraruminal administration was calculated as 90 min. This result is of the same magnitude as the half-life calculated for 5-methyloxazolidine-2-thione (5-MTO) (75 min.)

During the experiment (0-13 h post-infusion) 0.9 mg (0.25% of the administrated dose) was excreted via the urine. In milk only 0.1 mg (0.03% of the dose) were recovered over the experimental period. These values are of the same magnitude as those reported previously for 5-MTO (0.85% and 0.03% in the urine and in the milk respectively)

Table I : Comparison between the 5-VTO and thyroxine (T_4) plasma levels of 13 cows (Farm I,II,III) before and after feeding turnips for three weeks.

Farm	Cow	before feeding		after feeding	
		5-VTO (µg/dl)	T_4 (µg/l)	5-VTO (µg/dl)	T_4 (µg/l)
I	1	3.0	10.2	25.0	10.3
	2	N.D*	10.9	21.2	10.1
	3	N.D	8.5	34.4	7.8
	4	N.D	7.1	25.9	14.6
	5	N.D	11.2	17.0	9.3
II	6	N.D	9.0	3.9	8.0
	7	N.D	9.5	3.0	7.7
	8	N.D	17.6	4.0	13.5
	9	N.D	12.3	2.0	9.3
	10	N.D	13.6	3.3	9.2
III	11	N.D	16.4	7.6	11.4
	12	N.D	8.1	6.8	8.5
	13	N.D	19.8	7.2	14.3

*N.D = not detected (≤ 0.5 ppb)

-Concentrations of 5-VTO in bovine serum after feeding of turnips (B. Campestris)

Although rapeseed meal is the most important source of 5-VTO in animal fodder the edible parts of Brassicaceae contain substantial amounts of progoitrin (0.5 - 1 ppm). The chronic ingestion of the small amounts of this natural thyreostatic drug with the normal fodder may affect the thyroid and thus invalidate histological and macroscopic methods used for the control of the illegal application of the thyreostatic drugs. Therefore the effect of feeding turnips (B. Campestris) on the 5-VTO and the thyroxine level of bovine serum was studied.

From 13 cows, housed in 3 farms (I,II,III), blood was taken before and after feeding turnips for three weeks. The serum was analysed for its 5-VTO and T_4-content (Table I). In one animal only (nr.1) a low residue level of 5-VTO was detected in serum before starting turnip feeding. After three weeks of turnip feeding appreciable 5-VTO levels were found in the serum of all the animals : highest residue levels were found in the serum of animals housed in farm I. In fig.2 a chromatogram of serum before and after turnip feeding is shown. The thyroxine levels (T_4) of serum, after feeding turnips, were significantly ($p \leq 0.05$; non parametric sign test) lowered.

-Optimalisation of the extraction procedure of 5-VTO from animal tissue

The extraction of 5-VTO from tissues was studied on samples, collected from a goat fed rapeseed meal. Meat (2 g) was extracted with methanol and ethylacetate (10 ml) as organic solvents by homogenization with an ultra-turrax and centrifugation. After complete evaporation of the solvent, the residue was taken up in distilled water (2 ml) and extracted with PMA solution (14). It was found that the reaction of goitrine with PFB-Cl, was inhibited. Possibly traces of the organic solvent, retained on the residue, are responsible for this phenomenon. The direct extraction of tissue with PMA solution failed due to homogenization problems of the tissue with cyclohexane. The best results were obtained by homogenization of 2 g meat with distilled water (5 ml) in a glass tube (Using polycarbonate or polypropylene tubes severe losses of 5-VTO were detected). After centrifugation at 2500 rpm (1000 g) 2 ml supernatant was extracted with PMA solution. A chromatogram of a thyroid and a meat (M. diaphragma) extract, prepared by the procedure described above, are given in fig. 3.

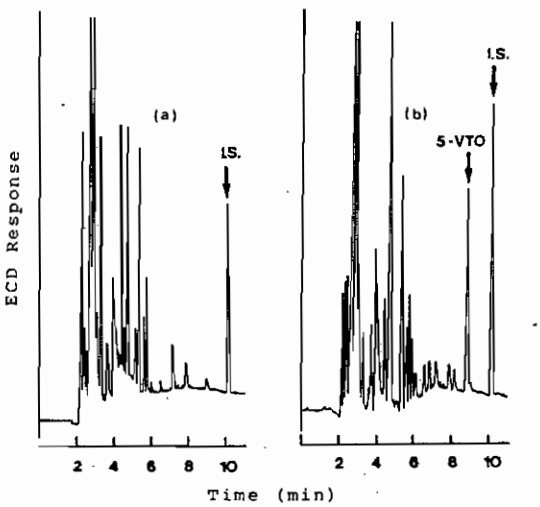

FIG 2 : Gaschromatogram of bovine serum
(a) before turnip feeding
(b) during turnip feeding (34 ppb 5-VTO)

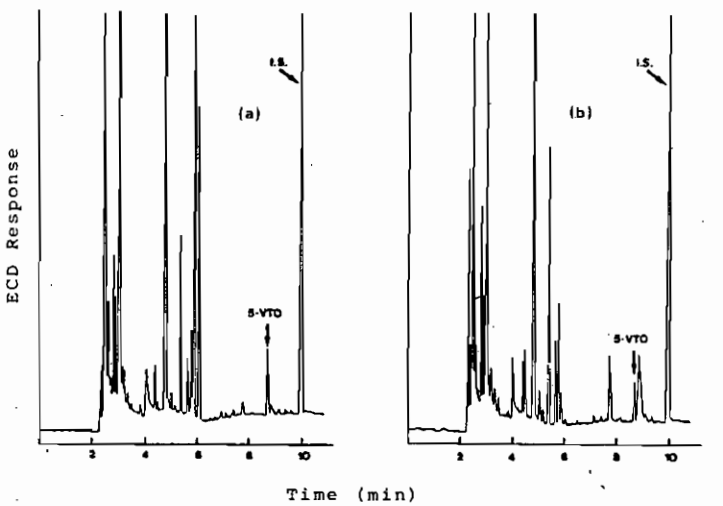

FIG.3 : Gaschromatogram of
(a) a thyroid extract (70 ppb 5-VTO)
(b) a meat extract (26 ppb 5-VTO).

-Determination of 5-VTO in goat tissues

A female goat (45 kg body weight) was fed twice daily (8h a.m. and 16h p.m.) with 50 g rapeseed meal (9.3 g/kg 5-VTO equivalents goitrine). The rapeseed meal was mixed with goat pellets containing no progoitrine. After each feeding the complete ingestion of the dosis was controlled. After 4 meals blood was taken (9h30 a.m.) and analysed as described previously (14). The 5-VTO concentration in plasma was 168 ppb. The next day (after 6 meals) the animal was slaughtered and blood, urine, milk, rumen fluid and tissue (thyroid, M. diaphragma, M.Longissimus dorsi, liver and kidney) samples were taken. The samples were analysed as described above and the results summarized in Table II. In the liver and the kidney of the goat no residues of 5-VTO were detected. In meat the 5-VTO concentration was of the same magnitude as found in plasma or milk. In urine and rumen fluid higher 5-VTO concentrations were found.

TABLE II: 5-VTO content of biological fluids and tissues of a goat after feeding rapeseed meal

fluid	5-VTO content (µg/kg)	tissue	5-VTO content (µg/kg)
plasma	22	thyroid	70
milk	25	M.Diaphragma	22
urine	101	M.Long.Dorsi	26
rumen fluid	48	liver, kidney	≤0.5

The results indicate a very fast equilibrium between plasma, milk and meat. In the thyroid, 5-VTO is concentrated, the 5-VTO concentration being 3 times higher than that of plasma. Also in urine and rumen fluid higher 5-VTO concentrations were found.

CONCLUSIONS

Rapeseed meal, containing substantial amounts of the 5-VTO precursor progoitrine (up to 1%) and used as a cheap protein supplement in animal feeds, may be considered as the main source of this thyreostatic drugs in animal feed. It is shown here that in the goat, following absorption, progoitrin is hydrolysed to 5-VTO in the rumen and appears in plasma, milk and meat. This thyreostatic drug is concentrated in the thyroid. Fortunately the half-life of oxazolidine-2-thiones in ruminants is small (70-90 minutes) so that residues do not accumulate. In contrast to earlier findings(13) the natural thyreostatic drug 1-5-vinyl-oxazolidine-2-thione (5-VTO) was also detected in tissues (meat, thyroid) of ruminants.

However,the health hazard, produced by the presence of these drugs in meat may be considered as negligible. For inspection on thyreostatic drugs however, the presence of progoitrine in animal feed will completely invalidate macroscopical (17, 18) and histological (19) methods. Different studies showed clearly that animals fed rapeseed meal have larger thyroids and score more histological positive results than control animals (20, 21). The thyreostatic effect, produced by these natural drugs may also influence the natural thyroxine (T_4) levels of ruminants and also invalidate these screening tests.

Thus, legislations should not allow to reject slaughter animals on basis of these "hypothyroid" symptoms only. Methods based on "hypothyroid symptoms" should only be considered as screening methods and rejection should only be based on the unequivocal detection of a "forbidden" drug.

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