# Evidence that urinary excretion of thiouracil in adult bovine submitted to a cruciferous diet can give erroneous indications of the possible illegal use of thyrostats in meat production

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#### Abstract

Thyrostats have been banned for use as veterinary drugs in Europe since 1981 because of their carcinogenic and teratogenic properties. Until now, the identification of thiouracil in animal biological matrices has been interpreted as the consequence of an illegal administration. The present paper studies the influence of a cruciferous-based feed on the occurrence of thiouracil as a residue in urine. Urine samples collected from two heifers fed on cabbage or rapeseed cakes were analysed for the presence of thiouracil by 3-iodobenzylbromide derivatization and liquid chromatography-electrospray ionization tandem mass spectroscopy (LC-ESI(–)-MS/MS) analysis. Urine collected after cabbage or rapeseed feeding showed thiouracil concentrations in the range 3-7 and  $2-9 \,\mu g l^{-1}$ , respectively, demonstrating a relationship between a diet based on cruciferous vegetables and the occurrence of thiouracil in urine. Thiouracil was excreted in urine in the hours following cruciferous intake. Complete elimination (< $0.8 \,\mu g l^{-1}$ ) of the compound occurred within 5 days. The precursors in cruciferous vegetables responsible for the thiouracil excretion in urine were proved not to be thiouracil itself.

**Keywords:** Thyrostats, thiouracil, urine, rapeseed, cabbage, cruciferous plants, liquid chromatography-electrospray ionization tandem mass spectroscopy (LC-ESI(-)-MS/MS)

#### Introduction

Thyrostatic compounds are orally active drugs that may be used to increase the weight of cattle before slaughter. This weight gain is mainly due to increased water absorption and retention within edible tissue as well as filling of the gastrointestinal tract by inhibiting thyroid hormone production (Kotter et al. 1959; Derblom et al. 1963). In particular, 2-thiouracil (TU) (Figure 1a), 6-methyl-2-thiouracil (MTU), 6-propyl-2-thiouracil (PTU) and 1-methyl-2-mercapto-imidazole (tapazole, TAP) are very strong thyroid-inhibiting compounds which are inexpensive and easy to obtain.

Correspondence: G. Pinel. E-mail: pinel@vet-nantes.fr ISSN 0265–203X print/ISSN 1464–5122 online © 2006 Taylor & Francis DOI: 10.1080/02652030600806370 The consequence of such abuse is not only the production of inferior meat quality, but also the potential risk of drug residues to human health. For these particular reasons, thyrostats have been banned within the European Union since 1981 (European Community 1981) for animal production. In consequence, reliable, sensitive and specific methods, which are mandatorily based on mass spectrometry according to the regulation (European Community 2002), have been set up to ensure efficient control of their illegal use (Le Bizec et al. 1997; De Wasch et al. 2001; Pinel et al 2005; Zhang et al. 2005). The newly developed protocols (Pinel et al. 2005) exhibit performances allowing detection



Figure 1. Tautomeric forms of 2-thiouracil (a) and the chemical structure of 2-thiouracil derivatized with 3-iodobenzylbromide (3-IBBr) (b).

and identification of the thyrostat compounds in biological fluids and edible tissues in the  $\mu g k g^{-1}$  or  $\mu g l^{-1}$  range, which is in accordance with the requirements of the European Union regarding the provisional minimum required performance limit (MRPL) fixed at  $100 \mu g l^{-1}$ . These methods allow unambiguous detection of thyrostats abuse since the concentrations that have to be administrated (5 g day<sup>-1</sup>) in order to observe the expected animal weight gain generate high urine concentrations ( $\gg 100 \mu g l^{-1}$ ).

Occasionally, however, the occurrence of thiouracil in bovine urine from the national control plan with quantifications in the range  $1-10 \,\mu g \, l^{-1}$  raises the question of the origin of the molecule, for example arising from low level abuse, the end of the kinetics of elimination, endogenous excretion or originating from animal feed. The latter hypothesis could be considered since some vegetables from the Cruciferae (Brassicaceae) family (Kennedy and Purves 1941) are known to contain substances called goitrogens that impair iodine uptake by the thyroid or impair its incorporation into thyroxine. These substances are called goitrogens and include thiouracil and thioglucosides. Thioglucosides or glucosinolates in particular are found in cruciferous vegetables (such as cabbage, cauliflower and rapeseed) (Tian et al. 2005). Glucosinolates constitute a well-defined group of secondary plant metabolites in cruciferous plants. Glucosinolates undergo

hydrolysis with the endogenous plant enzyme myrosinase (thioglucoside glucohydrolase EC 3.2.3.1) into a range of biological compounds. Enzymatic hydrolysis occurs after cell wall disruption caused by processes such as cutting, cooking or freezing (Verkerk et al. 1997). Very few studies deal with the natural occurrence of thiouracil in vegetables as a free compound or as an aglycone moiety (De Brabander 1984) and the influence of cattle feed on the presence of this compound in biological matrices has never been investigated up to now. To avoid false-positive indications of drug abuse, and since these vegetables are susceptible to be present in bovine feed through complementary nitrogen feeding stuffs, it is of major importance to elucidate this possible contribution from feed. Work aimed at identifying the thiouracil in bovine urine through different mass spectrometric evidence has been reported recently (Pinel et al. 2006) and was a necessary preliminary to the present work. The present paper aims to assess the influence of a cruciferous vegetable feed based on the concentration range for thiouracil in urine. Thiouracil determination was carried out according to a previous published method based on liquid chromatographyelectrospray ionization tandem mass spectroscopy (LC-ESI(-)-MS/MS) determination after 3-iodobenzylbromide derivatization (Pinel et al. 2005).

#### Materials and methods

#### Animal experiment

Two different experiments were conducted with heifers to evaluate the contribution of two cruciferous vegetables, cabbage and rapeseed, on the occurrence of thiouracil in urine upon consumption of these feeding stuffs.

Cabbages were collected directly from farms in January 2005; rapeseed cakes were obtained from a local agricultural cooperative store.

One heifer was fed with fresh cabbage twice a day, in the morning and afternoon, over 9 days from day zero ( $D_0$ ) to  $D_8$  (except on  $D_3$ ). Urine samples were collected during the same period twice a day, except on  $D_3$ .

The second experiment was conducted over 19 days, from  $D_0$  to  $D_{18}$ , with a different heifer from the one used in the first experiment. Feed was kept unchanged with hay for 7 days, then the heifer was fed with rapeseed cakes *ad libitum* for 4 days and finally returned to hay for 8 days. Urine samples were collected twice a day during this period.

For subsequent analysis of the vegetable matrices, cabbage leaves and stems were separated, grounded and freeze-dried.

### Chemicals

Standards were obtained from Sigma-Aldrich (St. Quentin Fallavier, France). Stock solution of 2-thiouracil (TU) was prepared in methanol at a concentration of  $100 \text{ ng }\mu\text{l}^{-1}$ . Working solutions were obtained by  $100 \times$  and  $1000 \times$  dilutions in methanol (1 and  $0.1 \text{ ng } \mu l^{-1}$ , respectively). The internal standard was synthesized in our laboratory: 2-benzyl-6-propyl-2-thiouracil (BPTU). Chemicals for extraction and purification steps were of analytical grade. Solvents for the preparation of the mobile phase were of high-performance liquid chromatography (HPLC) grade. They were from SDS (Peypin, France). all obtained Derivatization 3-iodobenzylbromide reagent (3-IBBr) was obtained from Sigma-Aldrich (St Louis, MO, USA) and prepared extemporaneously  $(2 \text{ mg ml}^{-1} \text{ in methanol})$ . Phosphate buffer, pH 8, was made up of 94.5 ml Na<sub>2</sub>HPO<sub>4</sub> 0.2 M and 5.5 ml KH<sub>2</sub>PO<sub>4</sub> 0.2 M.

# Material

Silica gel solid-phase extraction (SPE) disposable columns (1 g) were obtained from UCT (Bristol, PA, USA).

Separation of thyrostatic compounds was performed on a Nucleosil  $C_{18}$  AB column (5 µm, 2.5 × 50 mm) (Interchim, Montluçon, France) with an MeOH (A) and H<sub>2</sub>O containing 0.5% acetic acid (B) gradient, run at 300 µl min<sup>-1</sup> on an Alliance 2690 HPLC chromatograph (Waters, Milford, MA, USA). The gradient was linear as follows: A/B 30/70 to 70/30 from 0 to 20 min.

# MS measurements

A triple quadrupole mass analyser (Quattro-LC, Micromass, Manchester, UK) was used fitted with an ESI source operated in the negative mode. The analytes were detected using selected reaction monitoring (SRM) acquisition mode. The following transitions were monitored for BPTU and TU, respectively: 259>225, 58, 42 and 343>127, 95, 58, 42.

# Derivatization, extraction and clean-up

The analytical protocol describing the derivatization, extraction and purification of thyrostats from urine has been published elsewhere (Pinel et al. 2005). Briefly, thyrostats contained in urine were derivatized with 3-IBBr (pH 8, 1 h, 40°C) before extraction (Figure 1b). After a liquid/liquid extraction of the derivatized compounds (diethyl-ether, pH adjusted to 3 with HCl 35%), the samples were cleaned-up on silica cartridges (elution with a mixture of hexane/ethyl acetate 40/60) and injected

onto the LC column. Analysis of vegetables followed the same method with an additional solid/liquid extraction step at the beginning of the protocol. Methanol (5 ml) was added to 2 g of cabbage or rapeseed. The extraction proceeded for 30 min under agitation and the extract was obtained after centrifugation (3000g, 20 min). The methanolic extracts were dried under a gentle stream of nitrogen before the derivatization step occurred.

# **Results and discussion**

Cruciferous vegetables were chosen for this study because cabbage is historically the plant in which a goitrogen property is attributed to the presence of anti-thyroid agents (Kennedy and Purves 1941). Furthermore, cabbage is a plant on which cattle traditionally feed during the winter on farms. Rapeseed was chosen as it belongs to the Cruciferae family and is incorporated into animal feed as nitrogen source.

# Feeding and consequences on thiouracil concentrations in urine

All the urine samples collected during the different animal feeding experiments were analysed in order to establish the influence of feeding on thiouracil concentrations in urine. Urine samples arising from the experiment with cabbage feed were analysed for their content of thiouracil. Typical chromatograms are presented in Figure 2. The reference chromatogram (total ion current) shows the signals associated to the internal standard BPTU  $(100 \,\mu g \, l^{-1})$  eluted at 15.2 min and to thiouracil  $(10 \,\mu g l^{-1})$  with a retention time of 12.1 min (Figure 2a). The urine sample collected in the morning of the experiment  $(D_0 \text{ morning})$ , before the heifer had been provided with any cabbage, did not contain any free thiouracil shown on extracted ion chromatograms as in Figure 2(b). The extracted ion chromatograms of urine sample collected in the evening of day 5 (Figure 2c) provided unambiguous identification of thiouracil in the sample, with respect to number of monitored reactions, ion ratio and retention time criteria, according to European Decision 2002/657/ EC (European Community 2002) and as shown in previous work (Pinel et al. 2006). The concentration of thiouracil in the sample was found to be  $6 \mu g l^{-1}$ . The concentrations calculated in the different urine samples collected during the experiment are shown in Figure 3. These results clearly indicate that thiouracil appears in the urine samples within the first 10 h of the experiment ( $D_0$  evening). Thiouracil was always present in urine samples collected in the evening during the experiment and seldom in the morning samples. The highest concentration



Figure 2. Cabbage experiment. Ion chromatograms of (a) reference sample fortified with 2-thiouracil  $(10 \,\mu gl^{-1})$  and 2-benzyl-6-propyl-2-thiouracil (BPTU) as internal standard  $(100 \,\mu gl^{-1})$ , (b) a urine sample collected during the first morning of the experiment and (c) a urine sample collected on D<sub>5</sub> evening. Liquid chromatography-electrospray ionization tandem mass spectroscopy (LC-ESI(–)-MS/MS) analysis and selected reaction monitoring (SRM) acquisition mode.



Figure 3. Thiouracil elimination  $(\mu g l^{-1})$  in urine samples collected twice a day on a heifer fed on cabbage over 9 days.



Figure 4. Rapeseed experiment. Ion chromatograms of (a) reference sample fortified with 2-thiouracil  $(10 \,\mu g l^{-1})$  and 2-benzyl-6-propyl-2-thiouracil (BPTU) as internal standard  $(100 \,\mu g l^{-1})$ , (b) a urine sample collected during the first morning of the experiment and (c) a urine sample collected on D<sub>10</sub> evening. Liquid chromatography-electrospray ionization tandem mass spectroscopy (LC-ESI(–)-MS/MS) analysis and selected reaction monitoring (SRM) acquisition mode.

was reached on  $D_1$  in the evening  $(7 \mu g l^{-1})$ . According to these results, the correlation with a cabbage-based feeding and the occurrence of thiouracil in urine samples at concentrations between 5 and  $10 \,\mu g \, l^{-1}$  was established. To complete the results, it would have been of interest to check the absence of thiouracil in urine samples collected some evenings before the experiment with the feeding of cabbage started. A possible explanation for the occurrence of thiouracil in the evening urine samples could be the polar nature of the compounds present in the cabbage which is a water-based vegetable. Polar compounds such as thiouracil would be very easily assimilated and rapidly eliminated in urine. Indeed, thyrostats, and in particular thiouracil, are known to be rapidly eliminated in urine after oral administration (De Brabander 1984).

Urine samples collected during the rapeseed experiment were analysed for their content of thiouracil. Typical chromatograms are presented in Figure 4. As already shown with the cabbage experiment, thiouracil, which was not present in the urine samples collected before feeding with rapeseed cakes (extracted ion chromatograms shown in Figure 4b), appeared in the samples collected during the experiment (extracted ion chromatograms shown in Figure 4c). The calculated thiouracil concentrations in urine samples collected during the whole animal experiment are presented in Figure 5. In this experiment, no difference could be found between samples collected in the morning or in the evening. A possible explanation could be that rapeseed cakes are anhydrous stuffs and polar compounds are not released so rapidly as they are from aqueous stuffs such as cabbage. Three main periods can be distinguished: (1) urine collected during the 7 days before feeding with rapeseed did not contain any thiouracil; (2) urine collected during the feeding with rapeseed cakes in which thiouracil was identified in concentrations in the range  $1.5-7.5 \,\mu g l^{-1}$ , and (3) urine collected once the feeding with rapeseed cakes has been stopped and in which thiouracil was identified and quantified at concentrations as high as  $9 \mu g l^{-1}$ . This latter part showed a typical elimination kinetic behaviour since TU concentrations decreased with time to be undetectable ( $<0.8 \,\mu g \, l^{-1}$ ) 6 days after the end of the rapeseed feeding.

This second animal experiment confirmed the conclusions drawn after the first one as described



Figure 5. Thiouracil elimination  $(\mu g l^{-1})$  in urine samples collected twice a day (morning and evening) in a heifer fed on rapeseed cakes over 19 days.

above: a cruciferous-based feeding regime leads to the excretion and detection of thiouracil in urine of animals during the time of feeding. Concentrations were observed to be below  $10 \,\mu g \, l^{-1}$  and are in accordance with values sometimes reported for urine within the French national control plan. According to these results, which have never been reported before, the identification of thiouracil in urine samples at concentrations up to  $10 \text{ ug } \text{l}^{-1}$  should henceforth not lead automatically to the conclusion of illegal abuse of thiouracil. Results will have to be interpreted keeping in mind that thiouracil may possibly originate from feed. These recent observations of thiouracil at trace level certainly have to be linked to the use of new generation analytical instruments which have led to decreased detection limits and allowed measurement of decreased concentration levels previously undetectable due to older and less sensitive instruments.

To understand the origin of the urinary excretion of thiouracil, the composition of the feeding stuff used in this study was investigated, particularly focusing on similar chemical structures to that of thiouracil. Analysis of the different samples, cabbage leaves, stems and rapeseed cakes, did not show evidence of the presence of free TU.

These results raise the question of the origin of thiouracil in urine. The certain identification of thiouracil was a necessary preliminary, which has been achieved recently (Pinel et al. 2006) through different and independent mass spectrometric determinations (liquid chromatography coupled with tandem mass spectrometry detection (LC-MS/ MS), gas chromatography coupled with tandem mass spectrometry (GC-MS/MS), high-resolution mass spectroscopy (HRMS)) in bovine urine samples from animals never treated with any thyrostatic compounds. Even though thiouracil was identified unambiguously, the question of its origin remains unanswered. Further work is needed to exclude any analytical pitfall and precursor(s) identity has to be elucidated.

#### Conclusion

The present study allowed a correlation to be seen between the use of a cruciferous-based animal feed and the identification of thiouracil in the urine of animals through animal experiments and LC-MS/ MS analysis of the collected urine. In the future, this study, which is the first of its kind, will lead to a more careful interpretation of samples suspected of being 'non-compliant' with regard to thiouracil abuse. Concentrations of thiouracil eliminated in urine during the cruciferous diet were rather low and up to  $10 \mu g l^{-1}$ . Even though the relation between components in animal feed and thiouracil occurrence in urine has been established, neither free thiouracil nor its precursors have yet been identified in cabbage or rapeseed. Further work should be conducted in order to investigate the potential thiouracil-releasing compounds in vegetables. The discrimination between urine containing thiouracil of a natural origin or as a consequence of drug abuse should rely, for example, on potential and specific

phase I or phase II metabolites that will be searched for in the future. The use of isotopic methods such as Gas Chromatography-Combustion-Isotopic Ratio Mass Spectrometry (GC-C-IRMS) could also help in such discrimination.

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