Ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry detection of naturally occurring thiouracil in urine of untreated livestock, domesticated animals and humans

J. Vanden Busschea*, L. Vanhaecka, Y. Deceuninckb, K. Willea, K. Bekaerta, B. Le Bizecb and H.F. De Brabander

aGhent University, Faculty of Veterinary Medicine, Research Group of Veterinary Public Health and Zoonoses, Laboratory of Chemical Analysis, Salisburylaan 133, B-9820 Merelbeke, Belgium; bONIRIS, USC 2013, LABERCA Atlanpole-La Chantrerie, F-44307, Nantes, France

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Thiouracil belongs to the xenobiotic thyreostats, which are growth-promoting agents illegally used in animal production. Recently it has been reported that thiouracil is suspected to have a natural origin. The European Union of Reference Laboratories guidance paper of 2007 acknowledged this by stating that thiouracil concentrations below 10 μg l⁻¹ might have a natural origin derived from the consumption of Brassicaceae. The present research aimed at endorsing this possible natural occurrence. Urine samples of animals (livestock and domesticated) with known and unknown clinical backgrounds were analysed for thiouracil with a newly developed ultra-high performance liquid chromatography coupled to a triple quadrupole mass spectrometric analysis method without derivatisation. In addition, a small-scale 9-day human experiment with Brassicaceae vegetables was performed to investigate if this natural prevalence could be extrapolated to the human population. The untreated animals had thiouracil concentrations below 10 μg l⁻¹ acknowledging the alleged natural occurrence of thiouracil. As for the humans, in 66.7% of the urine samples thiouracil was found above the CC₁₀₀ of 2.2 μg l⁻¹. However, the correlation with the Brassicaceae diet proved to be non-significant (p = 0.095). Nevertheless, these results clearly demonstrate the natural occurrence of thiouracil in urine of animals and humans. The exact origin of this natural thiouracil trace still needs to be identified.

Keywords: liquid chromatography/mass spectrometry; clean-up; exposure; extraction; drug residues; hormones; animal feed; vegetables; processed foods; animal feedingstuffs

Introduction

Thiouracil (TU) belongs to the group of xenobiotic thyreostats. These are orally active drugs that upon administration disturb the normal metabolism of the thyroid gland by inhibiting the production of the hormones triiodothyronine and thyroxine (De Brabander 1984; Courtheyn et al. 2002). This goitrogenic activity may be attributed to the presence of a thiocarbamide group (Mackenzie and Mackenzie 1943). In livestock, the administration of thyreostats results in a considerable live weight gain, mainly caused by increased water retention in edible tissue and augmented filling of the gastrointestinal tract (Kotter et al. 1959; Derblom et al. 1963). Consequently, these growth-promoting agents negatively affect the meat quality of treated animals. In addition, some xenobiotic thyreostats such as TU are listed as compounds with teratogenic and carcinogenic properties and they thus pose a possible human health risk (International Agency for Research on Cancer (IARC) 2010). These arguments led in 1981 to a ban on the use of thyreostats for animal production in the European Union (European Community 1981).

In recent years questions have been raised with regard to the status of TU. The onset of this was given by Pinel et al. (2006), who reported a correlation between the supplementation of a Brassicaceae diet to cattle and the presence of TU in bovine urine. This was considered as a first indication that TU might have a natural origin. In December 2007 the European Union of Reference Laboratories (EURLs) acknowledged this possibility by posting a guidance paper stating a recommended concentration (RC) of 10 μg l⁻¹ for TU in urine (European Union of Reference Laboratories (EURLs) 2007). According to their opinion, all values below this RC could be linked to a natural origin. In general, for detecting TU in urine at these low-level concentrations, LC-MS analysis based on the protocol of Pinel et al. (2005) was performed. Prior to the analysis of TU, which is a small, amphoteric and relatively polar molecule, derivatisation with

*Corresponding author. Email: Julie.Vandenbussche@Ugent.be
3-iodobenzylbromide (3-IBBr) was conducted to aid in extraction and detection (Pinel et al. 2005; Lõhmus et al. 2009; Vanden Bussche et al. 2009). However, the use of a derivatisation step may lead to possible false-positive results. To this purpose, a U-HPLC-MS/MS analysis procedure was recently been developed that allowed the detection of TU in urine without derivatisation. This method proved able to detect TU in urine without derivatisation. This method proved able to detect TU in urine without derivatisation.

In addition, this newly developed method, without derivatisation, significantly reduced the likelihood of false-positive results, and it was designed to assist in investigating the natural occurrence of TU.

The aim of this study is to establish the presence of TU in urine of untreated livestock, i.e. in the low-level range (<10μg L⁻¹). Therefore, field samples of various species were analysed using the newly developed method to exclude possible false-positive results. If TU is detected in these field samples, it concerns most likely, as stated by EURL (2007), a contamination of natural origin derived from feed. This poses yet another question. Can this natural prevalence be extrapolated to the human population, and if so, is this then correlated with any dietary habits, possibly elucidating the natural origin? Therefore, this study also aimed at unravelling the presence of TU in human urine and it investigated a possible correlation with the administration of a Brassicaceae-containing diet. The generated data may also be of value for indirect risk assessment regarding the body burden of TU. To this purpose, a small-scale experiment with six healthy volunteers was conducted during which the volunteers were asked to consume Brassicaceae vegetables. The sampled urines were subsequently analysed for the presence of TU by means of ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry (U-HPLC-MS/MS) (Vanden Bussche et al. 2010).

Materials and methods

Reagents and chemicals

The chemical standard 2-thiouracil (TU) and deuterated internal standard 6-propyl-2-thiouracil-D5 (PTU-D5) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Toronto Research Chemicals, Inc. (Toronto, ON, Canada), respectively. Stock solutions were prepared in methanol at a concentration of 200 ng μL⁻¹. Working solutions were prepared by 200× and 2000× dilutions in methanol (1.0 and 0.1 ng μL⁻¹, respectively). When necessary, sonication was applied to ensure the complete dissolution of the substances. Solutions were stored in dark glass bottles at 7°C.

Reagents were of analytical grade when used for extraction, and of Optima® LC-MS grade when used for U-HPLC-MS/MS analysis. They were obtained from VWR International (Merck, Darmstadt, Germany) and Fisher Scientific (Loughborough, UK), respectively.

Phosphate buffer, dissolved in deionised water, was made up from 0.5 M Na₂HPO₄.2H₂O and 0.5 M KH₂PO₄, adjusted to pH 7. For extraction purposes the required amount of phosphate buffer, pH 7, was saturated with 1% of DL-dithiothreitol (DTT, purity 99%, Sigma-Aldrich, St. Louis, MO, USA).

Instrumentation

Separation of thyreostatic compounds was carried out at 35°C on an Acquity HSS T3 column (High Strength Silica particles) (1.8 μm, 100 mm × 2.1 mm, Waters, Milford, MA, USA), coupled to an Accela U-HPLC pumping system (Thermo Fisher Scientific, San José, CA, USA). In addition, an Acquity UPLC in-line filter (2.1 mm, 0.2 μm, Waters) was used to improve analytical column lifetime. The mobile phase consisted of 0.1% aqueous formic acid and 0.1% formic acid in methanol, and was pumped at a flow rate of 0.3 ml min⁻¹. Optimised separation of the analytes was obtained using a linear gradient starting with a mixture of 95% aqueous formic acid and 5% formic acid in methanol. After 1.65 min the amount of acidified methanol was increased to 100% in 5.2 min and kept there for 0.5 min. Finally, the column was allowed to re-equilibrate for 2 min at initial conditions, this before each run. Analysis was performed on a triple quadrupole mass analyser (TSQ Vantage, Thermo Fisher Scientific, San José, CA, USA), fitted with a heated electrospray ionisation (HESI II) source operating in positive-ion mode. The following working conditions were applied: spray voltage at 3.5 kV; vaporiser and capillary temperature at 370 and 300°C, respectively; sheath and auxiliary gas at 40 and 20 arbitrary units (a.u.), respectively; and cycle time of 0.8 s. Argon pressure in the collision cell (Q2) was set at 1.5 mTorr and the mass resolution at the first (Q1) and third (Q3) quadrupole was set at 0.7 Da at full width at half maximum (FWHM). Precursor ion, S-lens RF amplitude and collision energy (CE) in Q2 were optimised individually per compound (Table 1). Quantification and confirmation data for TU were acquired in the selected reaction monitoring (SRM) acquisition mode. The transitions followed for TU and PTU-D5 are also shown in Table 1. Instrument control and data processing were carried out by means of Xcalibur Software (2.0.7, Thermo Fisher Scientific, San José, CA, USA). Additionally, data were statistically interpreted using ANOVA.
Samples livestock and domesticated animals
The urine samples were divided into two groups. The first group comprised bovine \((n = 222)\), porcine \((n = 63)\) and ovine urine samples \((n = 19)\) obtained from veterinary sampling in light of the European residue control plan of Belgium and Norway. Upon arrival at laboratory, samples were stored at \(-20^\circ\text{C}\), and thawed before analysis. The second group consisted of animals (seven porcine, one bovine, one equine and one canine urine sample) housed at the Faculty of Veterinary Medicine (Ghent, Belgium), with a known clinical background. All samples, taken in a non-invasive manner by a veterinarian, were stored at \(-20^\circ\text{C}\).

Prior to analysis, the samples were thawed and subsequently centrifuged for 10 min at 4000 g; aliquots of 1 ml were used for the analytical procedure. To each sample, 50 ng of internal standard (PTU-D5) were added to obtain a final concentration of 50 \(\mu\text{g l}^{-1}\). As for the spiked samples, a standard solution (0.1 or 1.0 \(\mu\text{g l}^{-1}\)) of TU was added.

Human experiment
A small-scale 9-day study \((D_0-D_8)\), which consisted of a control and test period that every volunteer had to endure, was performed with healthy female \((n = 3)\) and male \((n = 3)\) volunteers aged 27–33 years. No pre-selection was conducted because a control period \((D_0-D_2)\) was included. During this period all volunteers were asked to refrain from any Brassicaceae vegetable or derivative (e.g. mustard and rapeseed oil) consumption to foresee a natural baseline level and obtain a certain degree of volunteer screening. No other restrictions were imposed on the diet. This experimental design was based on two different studies both dealing with the conversion of glucosinolates after ingestion of Brassicaceae vegetables (Getahun and Chung 1999; Rouzaud et al. 2004). During the test period \((D_3-D_5)\), processed Brassicaceae vegetables (e.g. cauliflower, broccoli and savoy cabbage) were provided to the volunteers and they were asked to consume a minimum of 150 g at dinner. This was followed by a 3-day control period \((D_6-D_8)\) during which the volunteers were asked for a second time to refrain from any Brassicaceae consumption as a follow-up to monitor the residual concentration of TU (Getahun and Chung 1999). Urine samples were collected twice a day by the volunteers, this during the whole experiment, and immediately frozen \((-20^\circ\text{C})\) upon sampling. This pilot study supplied commercially available food products in normal physiological quantities, and urine collection occurred in a non-invasive manner. Therefore, no authorisation from the medical ethical committee was required (Ross et al. 2007).

Sample clean-up and U-HPLC-MS/MS analysis
The analytical protocol describing the sample clean-up and U-HPLC-MS/MS analysis has been published elsewhere (Vanden Bussche et al. 2010). Briefly, TU contained in urine was reduced by 1% DTT at pH 7, this under denaturating conditions \((30\text{ min}, 65^\circ\text{C})\). Afterwards, a liquid/liquid extraction was performed with ethyl acetate. This was followed by evaporation under nitrogen and dissolution of the dried extract \((\text{A/B}, 90/10)\), with subsequent injection onto the U-HPLC system.

Results and discussion
Thiouracil in animal urine
In the framework of the national control plan of Belgium and Norway, our laboratory (Ghent University, Belgium) has frequently received urine samples from livestock for the routine analysis of thyreostats. These obtained urines, subsequently analysed, never exceeded the recommended concentration
for TU of 10 μg l⁻¹. Nevertheless, 61.3% of bovine urine samples had levels of TU below the RC; for porcine urine this was 96.3%; and for ovine urine 57.9% of the samples. The clinical background of these animals was unknown. Illegal administration for growth-promoting purposes however, seemed highly unlikely at these low concentrations (De Brabander 1984; Heeremans et al. 1998) and the possibility of a natural origin more plausible.

Next, urine sampled from different species with a thoroughly annotated clinical background, and no history of thyreostatic drug administration, was analysed. The experiment comprised seven porcine, one bovine, one equine and one canine urine sample. All samples, besides the urine originating from the mare, displayed traces of TU below 5 μg l⁻¹. TU was identified according to the criteria of retention time, monitored transitions, and ion ratios as set by EC/2002/657 (European Community 2002). Additionally, a co-chromatographic experiment was conducted to ascertain the presence of TU. Therefore, all untreated urines underwent a second analyses upon addition of TU at 5 μg l⁻¹ (Figure 1). When TU was added an increase in signal was observed at the retention time corresponding to TU, confirming the identity of the analyte. This implies that TU, i.e. below 10 μg l⁻¹, was detected in urine of different animal species that never received any thyreostatic treatment. Moreover, the collected urine samples were analysed with U-HPLC-MS/MS, significantly reducing the likelihood of possible false-positive results. Therefore, it may be concluded that TU has a natural origin, most likely originating from the feed source (Pinel et al. 2006). These data, because of the incompleteness of possible influencing parameters, such as age and sex of the animal, type of feed, were not investigated for

Figure 1. SRM chromatogram of (a) a bovine urine and (b) the same urine spiked with 5 μg l⁻¹ of thiouracil after U-HPLC-MS/MS analysis.
significant differences within the different species. Further research should however be performed to this purpose.

**Thiouracil in human urine**

After submitting all urine samples to U-HPLC-MS/MS analysis (Vanden Bussche et al. 2010), it became clear that TU was excreted by all healthy volunteers (Figure 2). In 66.7% of the samples, concentrations higher than the CC\textsubscript{a} value (2.2 \(\mu\)g l\(^{-1}\)) were reached. By including two blank periods in the beginning and at the end of the treatment period, during which volunteers were asked to refrain from Brassicaceae vegetables and derivatives consumption, it was intended to display the elimination kinetics of TU in human urine, as demonstrated by Pinel et al. (2006) in bovine urine. However, a correlation between the presence of TU in the urine and the Brassicaceae-rich diet could not be observed. Moreover, no significant differences in detected TU concentrations (\(p > 0.05\)) were found between the blank periods and the Brassicaceae period, although a \(p = 0.095\) was obtained. Additionally, it needs to be highlighted that the measured concentrations of TU for two male volunteers occasionally exceeded the recommended concentration of 10 \(\mu\)g l\(^{-1}\). Because of these unexpected results, co-chromatography was conducted on some samples, with spike levels of 5 \(\mu\)g l\(^{-1}\) of TU to confirm its presence (Figure 3). Moreover, six samples were reanalysed by LC-MS with the 3-IBBr derivatisation protocol (Pinel et al. 2005), by two different laboratories (Laboratory of Chemical Analysis, Ghent University, Ghent, Belgium; and LABERCA, ONIRIS, Nantes, France), which confirmed the obtained results and concentration levels. To the best of our knowledge this study is the first to report the presence of TU in human urine.

All six volunteers were healthy subjects with no record of a thyroid disease or thyreostatic treatment. Because of the high prevalence (66.7%) of TU in the analysed urines, the presence appears to be intake related. However, the contamination source comprised more than only Brassicaceae vegetables and derivatives, which was enforced by the presence of TU in urine derived from the blank period (D\(_0\)-D\(_2\)). The additional sources of the contamination could however not be identified. The above-mentioned results clearly indicate the natural origin of TU that was detected in human urine.

**Conclusions**

In this work the thyreostatic compound thiouracil (TU) was detected in the urine of untreated animal species, livestock as well as domesticated animals, but below the recommended concentration of 10 \(\mu\)g l\(^{-1}\). Traces of TU below the recommended concentration are accepted by the European Union of Reference Laboratory as contaminations of natural origin. In our study the alimentation between the animals differed and was not controlled, therefore the contamination source of these traces could not be identified.

Even more, the obtained results were transferable to the human population. A small-scale experiment indicated the presence of TU in human urine in 66.7% of the samples analysed. Noteworthy was the fact that the values for two male volunteers sometimes even exceeded the recommended concentration of 10 \(\mu\)g l\(^{-1}\). As for the contamination source, the Brassicaceae-rich diet did enforce the presence of the analyte, however in a non-significant way (\(p = 0.095\)). This provided a clear indication that a Brassicaceae diet was not the sole source of contamination for the naturally occurring TU detected in human urine.
Finally, from these results it can be concluded that the alleged xenobiotic thyreostat TU can occur naturally. Up till now its exact origin has remained unknown, but evidence points towards a nutritional origin. Future work will focus on the elucidation of this exact source. In light of this paper, another question that requires proper investigation has surfaced with respect to the impact of this low-level naturally occurring TU trace on the functionality of the thyroid gland. Does it affect the thyroid hormone profile and as such might it impose a possible health risk? Or, could TU possibly be a biomarker indicating a perturbed function of the thyroid gland? At this point these questions remain unanswered and further investigation regarding the body burden of naturally occurring TU is required.

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References

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