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ORIGINAL PAPER

Thyreostatic drugs, stability in bovine and porcine urine

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Abstract Thyreostatic drugs, illegally administrated to livestock for fattening purposes, are banned in the European Union since 1981. For monitoring their illegal use, sensitive and specific analytical methods are required. In this context, the knowledge of the stability in a matrix is of primary importance. This study aimed at evaluating the effects of preservation, number of freeze–thaw cycles, and matrix-related variables on the stability of thyreostatic drugs in the urine of livestock. Finally, the developed conservation approach was applied on incurred urine samples, which displayed traces of the thyreostat thiouracil below the recommended concentration of 10 μg L^{-1}. The stability study confirmed the negative influence of preservation (8 h) at room temperature and at −70 °C, decreases in concentration of more than 78.0% were observed for all thyreostats, except for 1-methyl-2-mercaptoimidazole and 2-mercaptobenzimidazole. Additionally, investigation of matrix-related variables indicated significant impacts of the presence of copper (p= 0.001) and the pH (p=0.002). Next, an optimised pre-treatment (pH 1 and 0.1 M ethylenediaminetetraacetic acid disodium salt dehydrate) significantly differing from the original conservation approach (p<0.05) was developed, which proved capable of delaying the decrease in concentration and improved the detection in time for both spiked as well as incurred urine samples. In the future, it seems highly advisable to apply the developed pre-treatment on incurred urines upon sampling, before thyreostat analysis. Additionally, it is recommendable to limit preservation of urine samples at room temperature, but also in the freezer prior to thyreostat analysis.

Keywords Thyreostatic drugs · Stability · Urine · UPLC · Mass spectrometry

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Introduction

Xenobiotic thyreostats are orally active drugs, which upon administration disturb the normal metabolism of the thyroid gland by inhibiting the production of the hormones triiodothyronine and thyroxine [1, 2]. This goitrogenic activity may be attributed to the presence of a thiocarbamide group [3]. In particular, 2-thiouracil (TU), 6-methyl-2-thiouracil (MTU), 6-propyl-2-thiouracil (PTU), 1-methyl-2-mercaptoimidazole (TAP, tapazole), and 2-mercaptobenzimidazole (MBI) display this strong thyroid-inhibiting action. 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 [4, 5]. Consequently, these growth-promoting agents negatively affect the meat quality of treated animals. In addition, xenobiotic thyreostats are listed as compounds with teratogenic and carcinogenic properties

and thus pose a possible human health risk (International Agency for Research on Cancer) [6]. These arguments led in 1981 to a ban on their use for animal production in the European Union [7].

In light of the residue control plan, which must ensure the elimination of thyreostat abuse, European legislation demands its member states to develop confirmatory methods to detect and quantify thyreostatic compounds in various biological matrices (e.g. urine, faeces, meat, thyroid gland, etc.). As a guideline for these methods, the European Union set out a provisional minimum required performance limit (MRPL) of 100 μg L^{-1} or μg kg^{-1}. To achieve this, new confirmatory methods have been developed and ameliorated over time, as reviewed by Vanden Bussche et al. [8]. Nowadays, the most common method used consists of a 3-iodobenzylbromide (3-IBBr) derivatisation followed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) [9]. Recently, this application was transferred to the new high-end technology of ultra-high performance liquid chromatography coupled to tandem MS (UPLC-MS/MS) [10]. Both methods comply with the MRPL, and even with the recommended concentration (RC) of 10 μg L^{-1} or μg kg^{-1}, as set out by the European Union Reference Laboratories in 2007 [11]. To ensure that detection methods applied in residue control are sufficiently robust and repeatable in time, the Commission Decision 2002/657/EC stated technical guidelines and performance criteria for validation of analytical detection methods [12]. During this procedure parameters like selectivity, specificity, linearity, trueness, recovery, applicability, ruggedness, repeatability, reproducibility, decision limit (CC_α) and detection capability are investigated. Imperative for the ruggedness of samples and their analytical results is also the knowledge of the stability of an analyte in a matrix. In many cases, this part of the validation study is reduced to a minimum or even omitted.

During routine analysis for residue control, occasionally non-compliant samples of forbidden substances (group A, Annex I of CD 96/23/EC) [13] have been reported, in which case a second analysis, performed at another laboratory can be requested. For thyreostatic drugs however, this confirmatory analysis has not always shown to be straightforward. In 2004 and 2006, when re-analysing non-compliant urine samples, the European Union Reference Laboratory (RIKILT, the Netherlands) reported a significant decrease in detected thyreostat concentration. This decrease may be ascribed to the lack of stability of thyreostats in matrix. In this context, the knowledge of the stability of an analyte is essential. Even more, it may support anomalous findings obtained during re-analysis.

Therefore, this study aimed at determining the stability of thyreostatic drugs in urine of livestock. Parameters such as preservation time, amount of freeze–thaw cycles, and stability at room temperature were investigated. Subsequently, a study to determine the kinetics and possible causes of the decrease in concentration was performed. These observations led to the development of a conservation approach, for which experiments, on a short and long-term basis were set-up on spiked urine samples of bovine and porcine origin by RIKILT (Institute of Food Safety, Wageningen University, the Netherlands). After establishing a pre-treatment (pH=1 and 0.1 mL of 0.1 M EDTA), the effectiveness of this procedure was tested upon incurred urine samples (bovine and porcine) at the Laboratory of Chemical Analysis (LCA, Ghent University, Belgium). For the incurred samples, in particular, the stability of TU was investigated. This analyte has drawn a lot of attention lately because of its possible endogenous status and its presence in urine of untreated livestock animals at concentrations below 10 μg L^{-1} [8, 14, 15]. Therefore, the investigation of the stability of low-level thiouracil (<10 μg L^{-1}) in incurred urine samples was considered as an important additional aspect within this stability study. To this end, all incurred urines were, upon sampling, divided into two aliquots, one remained unaltered, the second acidified and supplemented with EDTA. Again, the effect of freeze–thaw cycles and preservation time was investigated.

Material and methods

Reagents and chemicals

Standards were obtained from Sigma-Aldrich (St. Louis, MO. USA). Stock solutions of the thyreostatic drugs: TU, 6-benzyl-2-thiouracil (BTU), 6-dimethyl-2-thiouracil (DMTU), 6-ethyl-2-thiouracil (ETU), MTU, PTU, 6-phenyl-2-thiouracil (PhTU), TAP and MBI were prepared in methanol at a concentration of 200 ng μL^{-1}. Working solutions were prepared by 200× and 2,000× dilution in methanol (1 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. For the internal standards DMTU or ETU, spike levels of 5 and 10 μg L^{-1}, respectively, were used.

Reagents were of analytical grade when used for extraction and purification steps, and of Optima® MS grade for MS application, which were obtained from VWR International (Merck, Darmstadt, Germany) and Fisher Scientific UK (Loughborough, UK), respectively.

The derivatisation reagent, 3-iodobenzylbromide (3-IBBr, Sigma-Aldrich, St. Louis, MO, USA), was prepared extemporaneously (2.5 or 2 mg mL^{-1} in 1 mL methanol for RIKILT and LCA, respectively). As for the chelating agent, a 0.1 M solution of ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA) was prepared in water (Sigma-Aldrich, St. Louis, MO, USA).

Phosphate buffer, made up of 0.2 M Na_2HPO_4 and 0.2 M KH_2PO_4 in deionised water, was controlled and adjusted to a pH of 8.

Instrumentation

The samples were analysed at two different locations, RIKILT (Institute of Food Safety, Wageningen University, the Netherlands) and Laboratory of Chemical Analysis (LCA, Ghent University, Faculty of Veterinary Medicine, Belgium). Both laboratories used liquid chromatography coupled to mass spectrometry, UPLC-MS/MS and LC-MS^2, respectively. The outcome of a proficiency test for the detection of thyreostatic drugs in porcine urine (organised by the European Union Reference Laboratory), in 2008, ensured a good comparability between both 3-IBBR derived detection methods, as indicated by the highly satisfactory *z*-scores ($-2<x>2$) of both laboratories.

UPLC-MS/MS

Separation of the thyreostatic compounds was achieved at 60 °C on a Acquity UPLC BEH C18 column (1.7 μm, 100 mm×2.1 mm, Waters, Milford, MA, USA) with as mobile phases water containing 0.5% acetic acid (A) and acetonitrile (B), run at 0.25 mL min^{-1} on a Waters Acquity Ultra Performance system (Waters, Milford, MA, USA). The linear gradient was as follows: A/B 60/40 for 2 min, increased the amount of acetonitrile during 7 min to 15/85, and allowed the column to re-equilibrate for 3 min at 60/40. A Micromass Quattro Ultima Pt. mass spectrometer (Waters, Micromass, Manchester, UK) was used, fitted with an electrospray ionisation source operating in the positive ion mode. The following working conditions were applied: capillary voltage at 3.4 kV and a cone voltage of 40–70 V; source and desolvation temperature at 110 and 350 °C, respectively; cone and desolvation gas flow at 112 and 742 Lh^{-1}, respectively; cycle time of 0.39 s and a collision cell pressure of $1.90e^{-3}$ mbar [16]. The transitions monitored are displayed in Table 1.

LC-MS^2

Separation of the thyreostatic compounds was achieved at 30 °C on a Symmetry C18 column (5 μm, 150 mm× 2.1 mm, Waters, Milford, MA, USA) with as mobile phases water containing 0.5% acetic acid (A) and methanol (B), run at 0.30 mL min^{-1} on a Finnigan Surveyor LC-system (Thermo Electron, San Jose, USA). The linear gradient was as follows: A/B 50/50 for 3 min, increasing the amount of methanol during 17 min to 0/100, and allowed the column to re-equilibrate for 10 min at 50/50. A linear ion trap mass spectrometer (LTQ, Thermo Electron, San Jose, USA) was used, fitted with a heated electrospray ionisation source operating in the negative ion mode, except for TAP which required the positive ionisation mode. The following working conditions were applied: source voltage at 5 kV; vaporizer and capillary temperature at 250 and 275 °C, respectively; sheath and auxiliary gas at 30 and 5 arbitrary units (a.u.), respectively. The transitions monitored are displayed in Table 2.

Samples

For the experiments performed at RIKILT, blank bovine and porcine urine samples from healthy animals were provided by Animal Science Group (Lelystad, the Netherlands).

At LCA incurred urine samples, for investigating the stability and effect of the developed pre-treatment on low-level TU, were obtained from veterinary sampling in light of the European residue control plan. Upon sampling, these urines were divided in two aliquots, one remained unaltered, the second one acidified with hydrochloric acid (pH 1) and supplemented with 0.1 M EDTA (0.1 mL).

All samples were stored at −20 °C, and thawed before analysis. The thawed samples were than centrifuged for 10 min at 4,000×*g*, and aliquots of 1 mL were used for analysis. To each sample, 5 or 10 ng of internal standard (DMTU or ETU) was added, to obtain a final concentration of 5 or 10 μg L^{-1}, respectively. As for the spiked samples, a

Table 1 Monitored transitions per individual thyreostatic drug for the UPLC-MS/MS method (RIKILT, Wageningen University, the Netherlands)

Analyte	$[M+H]^+$	Product ion 1 (Coll. En.)	Product ion 2 (Coll. En.)	Retention time (min)
Tapazole	331	217 (16)	90 (30)	1.66
Thiouracil	345	217 (14)	90 (30)	3.31
Methylthiouracil	359	217 (14)	90 (30)	4.29
Mercaptobenzimidazole	367	217 (14)	90 (30)	4.51
Dimethylthiouracil	373	217 (14)	90 (30)	5.28
Propylthiouracil	387	217 (16)	90 (30)	6.30
Phenylthiouracil	421	217 (16)	90 (30)	6.60
Benzylthiouracil	435	217 (14)	90 (30)	6.80

Table 2 Monitored transitions per individual thyreostatic drugs for the LC-MS^2 method (LCA, Ghent University, Belgium)

Analyte	$[M+H]^+$	$[M-H]^-$	Product ions	Collision energy (eV)	Retention time (min)
Tapazole	331		114, 217	33	2.30
Thiouracil		343	182, 215, 309	44	9.36
Methylthiouracil		357	196, 229, 323	43	11.27
Mercaptobenzimidazole		365	148, 237, 332	32	10.16
Ethylthiouracil		371	210, 243, 337	44	13.36
Propylthiouracil		385	257, 351	45	14.65
Phenylthiouracil		419	171, 291, 385	35	15.74

standard solution (0.1 or 1 ng μL^{-1}) containing all thyreostats was added.

Sample extraction and purification

The analytical detection method applied by RIKILT [16] and LCA were both based on the protocol of Pinel et al. [9], with minor modifications. After the addition of 4 or 5 mL phosphate buffer to 1 mL of urine, the solution was derivatised with 100 μL of a 3-IBBr solution (at a concentration of 5 or 4 mg per 2 mL of methanol for RIKILT and LCA, respectively) and placed at 40 °C during 1 h. Before the liquid/liquid extraction with either ethyl acetate (3× 5 mL) or diethylether (3×3 mL), the solution was adjusted to a pH value between 2 and 4. The combined extracts were finally evaporated under a gentle stream of nitrogen and redissolved in either 500 μL of 30/70 acetonitrile/water (RIKILT) or 120 μL 50/50 methanol/water (LCA). Prior to redissolving the extracts LCA conducted an additional Si-SPE step, according to Pinel et al. [9]. In both cases, 20 μL was injected onto the column.

Quality assurance

Prior to the sample analysis, a standard mixture of the target compounds was injected to check the operational conditions of the chromatographic devices. To every sample, a procedure internal standard (IS) was added at a concentration of 5 or 10 μg L^{-1} (DMTU or ETU, respectively), prior to extraction. Identification of the thyreostats was based on the retention time relative to the IS and on the ion ratios of the product ions, carried out according to the criteria described in Commission Decision 2002/657/EC [12]. After identification, the analytes concentration was calculated, for the quantitative LC-MS/MS method this was performed by means of a calibration curve (0–1–2–5–10–20–50–100 μg L^{-1}). The LC-MS^2 detection method however was a semi-quantitative method, therefore concentrations were assessed based on a blank urine spiked with a known concentration of the thyreostatic drugs (TU at 5 μg L^{-1}, TAP 25 μg L^{-1}, others at 2 μg L^{-1}). Area ratios were determined by integration of the area of an analyte under the specific chromatograms in reference to the integrated area of the internal standard.

Data analysis

Data processing was performed using Masslynx, Quanlynx V4.1 (RIKILT; Waters, Milford, MA, USA) and Xcalibur™ 2.0.7 software (LCA; Thermo Fisher Scientific, San José, USA). All data were statistically interpreted using 2-way ANOVA, using the statistical software package S-PLUS 8.0 (Seattle, WA, USA). The level of significance was set at 5%, where the null hypothesis will be rejected if the obtained p value is below the significance level of 0.05.

Results and discussion

Spiked urines samples

To determine the stability of thyreostatic drugs in urine and establish the matrix-related variables, analyses of spiked urine samples of bovine and porcine origin were performed by RIKILT. The thyreostatic drugs investigated, comprised TAP, TU, BTU, MTU, MBI, PTU, and PhTU and were spiked as a mixture. Different spike levels were used, individually chosen per experiment between the range of the MRPL (100 μg L^{-1}) and the RC (10 μg L^{-1}), to display the most beneficial kinetics.

Stability of thyreostats in urine

During a first experiment the stability of thyreostatic drugs was investigated in urine during preservation in the freezer (−70 °C). Aliquots of 1 mL of bovine and porcine urine, but also of water (n=4) were spiked at 20 μg L^{-1} and stored at −70 °C. Each sample underwent one, two, three of four freeze–thaw cycles with a defrosting period of 3 h during four consecutive days. A significant decrease in thyreostat concentration could be noted, which showed a correlation with the increase of number of freeze–thaw cycles, for TAP

this effect was less pronounced (Fig. 1). Additionally, a significant matrix effect of urine could be observed [17]. Indeed, it appeared that the urine matrix was responsible for the decrease in thyreostat concentration, since the concentration of analytes remained stable in water. Even more, the observed losses were more pronounced in bovine than in porcine urine (Fig. 1). Noticeable however was that upon addition of 3-IBBr derivatised thyreostats to blank urine, limited losses were observed during preservation. Moreover, with the incorporation of four freeze–thaw cycles, the losses of all analytes were below 10%, except for BTU and TAP, with 36.3% and 39.2% respectively. Whereas without derivatisation, losses between 54.8% and 97.4% were obtained, These results stress the stability of the derivatised thyreostats as opposed to the original underivatised compounds.

Study of the matrix-related variables

Besides the insight in the rate of decrease of thyreostat concentration in urine, the mechanism behind this noticeable decrease was of interest as well. Possible causes including pH, daylight, enzyme activity, salt, and metal ion concentration (Cu^{2+}) were kept under consideration [18, 19]. The above-described experiment ("Stability of thyreostats in urine") indicated a higher loss in bovine than in porcine urine. For this reason, subsequent experiments were conducted with bovine urine to simulate the 'worst-case scenario'.

To monitor the decrease in thyreostat concentration, bovine urine samples (10 mL) were spiked with all thyreostats at 20 μg L^{-1} and kept at room temperature for 8 h. Every hour, a 1-mL aliquot was taken, prepared, derivatised, and analysed. This experiment indicated a linear decrease in time of the thyreostatic drugs in bovine urine. A correlation coefficient (R^2), which represented the area ratio of the analyte plotted against its concentration level, higher than 0.91 was obtained for all analytes except for TAP, for which a R^2 of 0.82 was calculated. All analytes displayed a concentration decrease per hour of 10.0% to 12.2%, while for MBI this only amounted to 6.0%.

For investigating the matrix-related variables behind the decrease in thyreostat concentration, spiked (20 μg L^{-1}) urine aliquots of 1 mL were placed 8 h at room temperature, before conducting sample clean-up and UPLC-MS/MS analysis. Several variables were evaluated to this end, namely daylight vs. darkness, pH 1–3 vs. pH 7–8 vs. pH 12–14, boiling (8 min) to inhibit enzyme activity, 1 M NaCl and a blank. The effect of daylight, boiling, and salt addition proved not to be significant ($p>0.050$). The impact of the pH value however was significant ($p=0.002$), in particular for thiouracil and its analogues (Fig. 2). At lower pH values, the highest concentration was retrieved for all analytes. In general, the analytes remained stable at pH 1–3, only

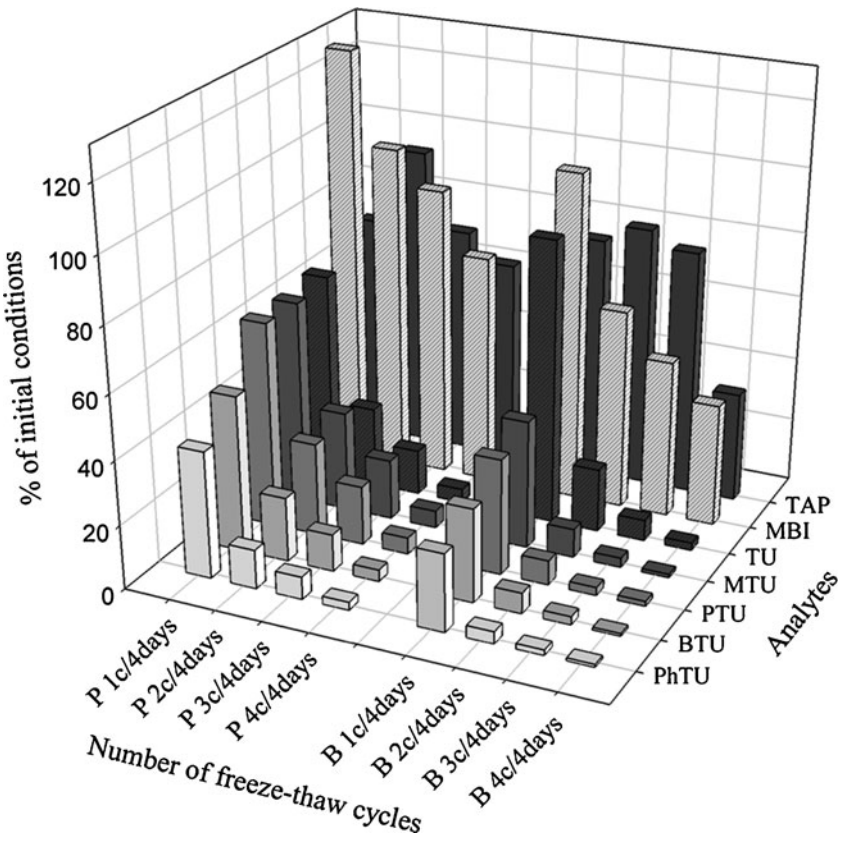

Fig. 1 The effect of the number of freeze–thaw cycles (one, two, three or four) in bovine (*B*) and porcine (*P*) urine on the thyreostat concentration (% of initial conditions), spiked at 20 μg L^{-1}, performed during four consecutive days

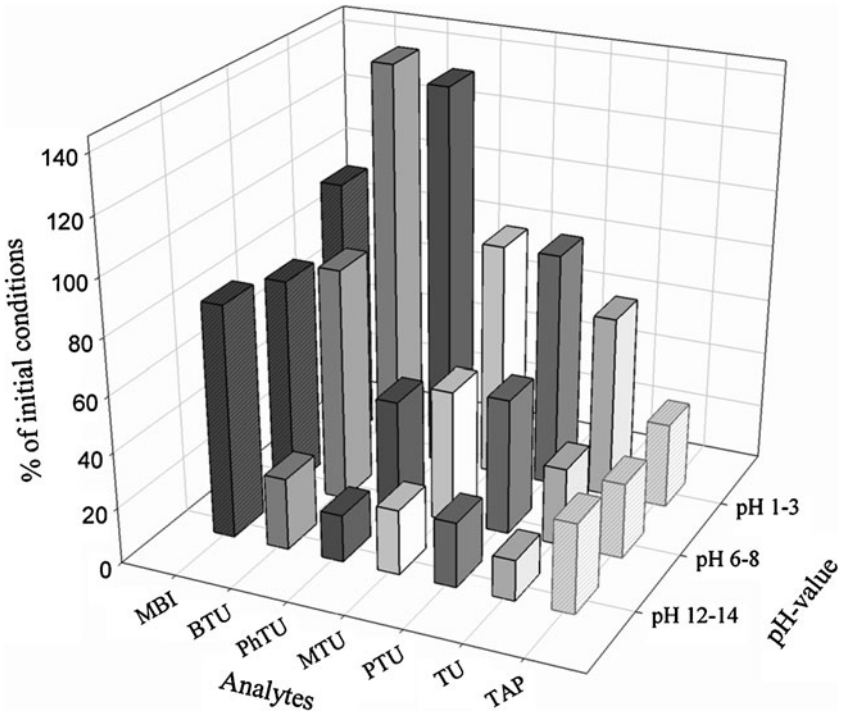

Fig. 2 The effect of different pH values on the thyreostat concentration (20 μg L^{-1}) in bovine urine when left 8 h at room temperature before clean-up, 3-IBBr derivatisation, and UPLC-MS/MS analysis

thiouracil still displayed losses up to 35%. The effects of 1 M $CuSO_4$ and 1 M Na_2SO_4 were investigated in water samples to exclude the interfering effect of ions inherent to urine. The addition of copper (II) caused a significant decrease (p=0.001) in analyte concentration, for thiouracil and analogues a loss of more than 80% was noted, for TAP and MBI this amounted around 50%. The interaction between the thiol group of the thyreostats and copper ions or other metal ions is generally well known [1, 19–21].

Increasing stability of thyreostatic drugs in urine

Subsequent experiments were intended to elucidate of the parameters that would maintain or prolong the stability of thyreostats in urine during preservation and also to investigate the effectiveness of the possible pre-treatment steps. The above-described experiments ("Study of the matrix-related variables") demonstrated that a pH value greater than 3 and the presence of copper significantly influenced the loss of thyreostatic drugs in urine. For this reason, the pre-treatment consisted of a fixed pH value of 1 and the addition of the chelating agent EDTA, which inhibits the interaction between copper ions and thyreostats by scavenging these ions.

To investigate the effectiveness of this pre-treatment, additional experiments with freeze–thaw cycles were conducted. Four blank bovine urines (5 mL) were set at pH 1, 7, 13 or supplemented with 0.1 M EDTA (0.1 mL). Each pre-treated sample was divided in 4 aliquots of 1 mL, and subsequently spiked at 100 μg L^{-1} with a mixture of seven thyreostats and internal standard. These samples were frozen at −70 °C and underwent one, two, three or four freeze–thaw cycles (defrosted during 3 h) during four consecutive days. This experiment demonstrated that the thyreostatic concentration was more stable at pH 1 and that the addition of EDTA also assisted in the process (Fig. 3) [18].

Next, the stability of thyreostats in pre-treated urine (pH 1 and 0.1 mL of 0.1 M EDTA) was compared to untreated urine, this on a short and long-term basis. For the short-term stability, the losses in thyreostatic concentrations at room temperature were followed up during 24 h. To this extent, ten different bovine urines were selected (20 mL each), and divided in two aliquots of 10 mL, one portion unaltered, the other set at pH 1 and supplemented with 0.1 mL of 0.1 M EDTA. Spike levels were set at 50 μg L^{-1} and 1 mL aliquots were taken, prepared, derivatised and analysed at 0, 2, 4, 6, 8 and 24 h. The results demonstrated that the pre-treatment of pH 1 and addition of EDTA stopped or delayed the decrease of thyreostats for 24 h at room temperature, with the exception of TAP for which a significant loss was observed between 8 and 24 h (Table 3). To evaluate the long-term stability, bovine urine samples were frozen and analysed once every month, this during 3 months. Again untreated urine was compared to pre-treated urine (pH 1 and EDTA), but also storage time versus the number of freeze–thaw cycles were included as variables in the set-up. To this end, bovine urine (untreated and pre-treated) was either frozen in bulk and underwent the freeze–thaw cycles in bulk, or was frozen in separate aliquots of 1 mL that only endured one freeze–thaw cycle. During this long-term stability experiment samples were prepared, derivatised and analysed after 0, 1, 2 and 3 months.

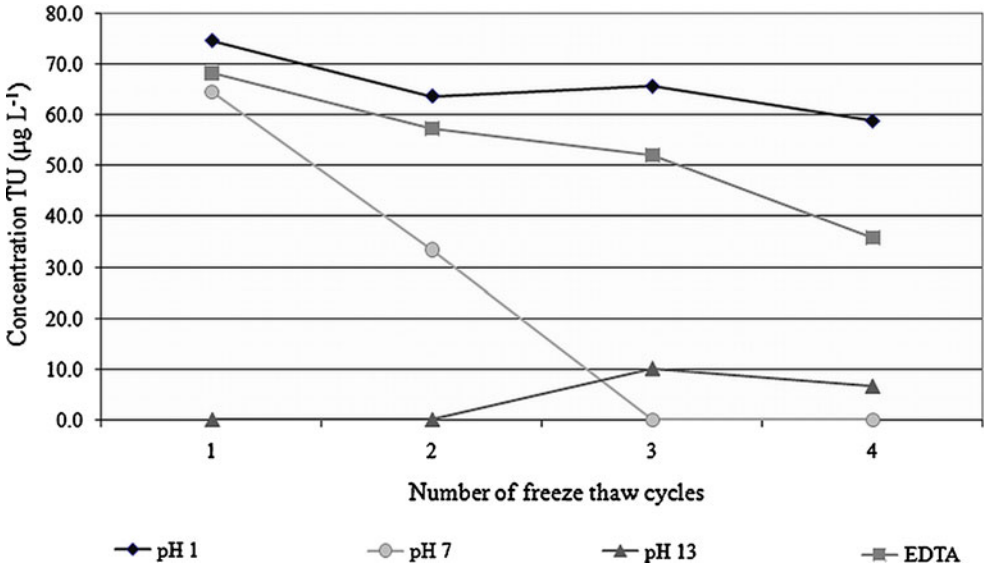

Fig. 3 The effect of different pre-treatments on the concentration (in micrograms per liter) of the thyreostatic drug, thiouracil in bovine urine upon storage at −70 °C and upon exposure to a number of freeze–thaw cycles, before UPLC-MS/MS analysis

No significant difference ($p>0.050$) was observed between the behaviour of bulk urines or separate aliquots (Table 4). The number of freeze–thaw cycles on a long-term basis proved to be of lesser significance than the time of preservation. As for the effect of pre-treatment, setting the bovine urine at pH 1 and adding 0.1 mL of 0.1 M EDTA clearly delayed the losses in thyreostat concentrations for 3 months at −70 °C. TAP again formed an exception, although its decrease in concentration was delayed in common with the other thyreostats, after 3 months the concentration of the pre-treated urine was similar to that of the untreated sample.

Incurred samples

In light of the national control plan, LCA (Ghent University, Belgium) performed LC-MS^2 analyses of incurred urine samples for thyreostatic drugs. In the event of non-fraudulent action, residue analysis of these samples should lead to compliant results. In case residue traces are detected, their concentrations should be below the RC of 10 μg L^{-1}. Nevertheless, these compliant samples could still be considered as positive, if the detected residue concentration is higher than the decision limit (CC_α) and when all criteria are fulfilled as stated in EC/2002/657 [12]. In recent years, many compliant urines samples were found positive for thiouracil at concentration levels below the RC [8, 10, 14], which in turn has raised the question of a semi-endogenous status of thiouracil [14, 15]. For this reason, only results of thiouracil could be presented in this section, since all other thyreostats monitored were absent. For residue analysis, it is also of importance to observe the behaviour and stability of low-level thiouracil (<10 μg L^{-1}) [11] in urine and investigate the benefit and necessity of conducting a pre-treatment (pH=1 and EDTA). In this section, the behaviour of thiouracil in bovine and porcine urine was not compared, because the number of urines was too limited. The urine samples, bovine ($n=13$) as well as porcine ($n=10$) were supplied in two aliquots to our laboratory, one untreated and one pre-treated (pH 1 and 0.1 mL of 0.1 M EDTA). Immediately upon arrival, 1 mL of each aliquot of all samples was prepared, derivatised, and analysed by LC-MS^2. The remainder of the aliquots was divided in a smaller portion (2 mL), which was subsequently stored at −20 °C and reanalysed after 5 months of preservation, and a larger portion (10 mL). The latter was also stored at −20 °C, but reanalysed every month during 5 months to monitor the effect of the number of freeze–thaw cycles.

The analysis upon arrival, already indicated a beneficial effect of the pre-treatment (pH 1 and 0.1 mL of 0.1 M EDTA), since TU was detected in a higher number of samples after application of this pre-treatment and this was true for both species (bovine and porcine; Table 5). The trend was even more pronounced for the bovine samples, as besides the higher number of TU-positive samples the pre-treated samples also displayed higher TU concentrations. To investigate the effect of the preservation time, all aliquots (10 mL) from bovine and porcine origin, untreated and pre-treated were reanalysed five additional times at 1, 2, 3, 4 and 5 months. A large decrease in TU concentration, both for untreated as well as for pre-treated urine, was noted after 1 month of preservation. Nevertheless, a significant difference ($p<0.0001$) could be observed in TU concentration between the untreated and pre-treated samples. Even more, the pre-treatment of urine (pH 1 and EDTA) prolonged the detection of TU in time (Fig. 4). Additional analyses were performed to investigate the effect of the number of freeze–thaw cycles, this on the aliquots, who were preserved during 5 months (−20 °C) without any freeze–thaw cycles. In this case however, significant differences ($p>0.050$) could be

Table 3 Short-term stability of thyreostatic drug concentration (in micrograms per liter±S.D.) in bovine urine (n=10) compared to identical urines with a pre-treatment (pH 1 and 0.1 mL of 0.1 M EDTA), both spiked at 50 μg L^{-1} and kept for 0, 2, 4, 8 and 24 h at room temperature before sample preparation and UPLC-MS/MS analysis

Analyte	Untreated						Pre-treated					
	t_0	t_2	t_4	t_6	t_8	t_{24}	t_0	t_2	t_4	t_6	t_8	t_{24}
Tapazole	22.1±6.5^{a}	23.0±3.2^{a}	16.4±2.5^{abc}	17.0±5.0^{ab}	12.8±3.9^{bc}	9.1±8.5^{c}	20.2±6.1^{d}	22.9±9.5^{d}	22.7±8.6^{d}	19.8±8.1^{d}	21.0±8.1^{d}	15.5±16.3^{d}
Thiouracil	15.4±5.4^{a}	9.3±4.1^{b}	4.4±3.7^{c}	2.5±2.6^{c}	1.3±1.5^{c}	0.3±0.4^{c}	12.2±5.8^{d}	12.3±4.5^{d}	14.5±5.4^{d}	13.0±5.9^{d}	13.7±6.5^{d}	14.8±6.8^{d}
Benzylthiouracil	20.1±5.8^{a}	10.5±4.9^{bc}	7.2±4.9^{c}	3.6±3.9^{cd}	1.7±1.7^{cd}	0.8±0.9^{d}	23.0±7.2^{e}	25.4±10.4^{e}	20.4±10.6^{e}	24.6±9.1^{e}	22.2±11.7^{e}	23.7±6.0^{e}
Methylthiouracil	19.3±5.0^{a}	14.7±2.8^{b}	8.3±3.7^{c}	5.2±3.6^{cd}	2.9±2.4^{de}	0.7±1.1^{e}	14.8±5.4^{f}	14.3±4.2^{f}	16.8±4.6^{f}	16.2±5.5^{f}	16.8±6.3^{f}	17.1±6.5^{f}
Mercaptobenzimidazole	28.5±7.1^{a}	27.5±4.6^{ab}	19.5±5.7^{bc}	15.6±6.1^{cd}	11.6±6.0^{cd}	7.9±8.8^{d}	35.2±5.7^{e}	37.0±6.9^{e}	38.7±8.7^{e}	36.1±8.8^{e}	30.6±9.1^{e}	26.9±16.3^{e}
Propylthiouracil	20.7±4.1^{a}	15.1±6.1^{b}	9.6±5.7^{c}	5.5±4.5^{cd}	2.9±2.8^{d}	0.8±1.1^{d}	16.9±4.8^{e}	18.7±5.6^{e}	18.5±5.0^{e}	19.2±5.2^{e}	17.4±4.7^{e}	18.9±4.2^{e}
Phenylthiouracil	19.9±6.4^{a}	7.9±3.9^{b}	5.2±4.2^{bc}	2.6±2.8^{c}	1.3±1.2^{c}	0.7±0.7^{c}	26.8±9.2^{d}	29.3±13.4^{d}	23.9±15.1^{d}	30.3±12.1^{d}	27.7±16.3^{d}	26.6±6.2^{d}

$^{a, b, c, d, e, f}$: Means within a row with a different superscript differ (p value<0.05)

Table 4 Long-term stability of thyreostatic drug concentration (in micrograms per liter) in bovine urine, with individual portions compared to bulk additional to the untreated versus pre-treated portions (pH 1 and 0.1 mL of 0.1 M EDTA), with a spike level of 100 μg L^{-1} and kept during 3 months at −70 °C

Analyte	Untreated (μg L^{-1})								Pre-treated (μg L^{-1})							
	Portion				Bulk				Portion				Bulk			
	t_0	t_1	t_2	t_3	t_0	t_1	t_2	t_3	t_0	t_1	t_2	t_3	t_0	t_1	t_2	t_3
Tapazole	147.8	25.3	6.8	34.4	175.8	11.0	7.5	11.9	120.9	80.1	37.7	52.7	118.3	92.9	49.5	41.9
Thiouracil	93.6	15.9	0.8	9.4	102.0	3.3	0.8	0.0	76.7	71.3	46.9	48.9	68.0	87.6	58.6	72.5
Benzylthiouracil	90.7	26.6	4.2	34.1	92.5	13.3	4.8	6.0	114.1	99.3	55.7	56.1	113.1	105.5	86.1	84.8
Methylthiouracil	89.4	52.7	9.0	33.5	88.6	30.3	11.9	6.4	74.5	71.9	50.1	48.8	66.1	73.4	60.4	63.5
Mercaptobenzimidazole	65.2	1.8	0.4	4.4	71.3	0.5	0.5	1.6	83.4	82.6	27.2	63.2	74.5	61.5	41.8	48.3
Propylthiouracil	77.2	48.0	9.2	43.4	78.6	28.6	12.8	9.1	64.3	60.8	59.4	59.3	66.3	70.7	73.5	74.7
Phenylthiouracil	90.7	26.6	4.2	38.9	92.5	13.3	4.8	6.4	114.1	99.3	55.7	76.0	113.1	105.5	86.1	102.0

At 0, 1, 2 and 3 months samples were prepared for UPLC-MS/MS analysis

Table 5 LC-MS^2 analysis upon arrival (t=0 months) of the incurred urine samples for low-level thiouracil concentration, comparing untreated to pre-treated (pH 1 and 0.1 mL of 0.1 M EDTA) urine samples

Thiouracil (3-IBBr)	Bovine (n=13)		Porcine (n=10)	
	Untreated	Pre-treated	Untreated	Pre-treated
Treated number of samples positive for TU	3	5	7	9
Mean concentration (μg L^{-1})	1.1±0.1	5.1±3.0	2.4±0.8	2.5±1.4

detected in TU concentration as compared to an identical aliquot that underwent six cycles during 5 months at −20 °C. Even more, the results displayed a pernicious effect of long-term storage for TU detection in urine, independent of the number of freeze–thaw cycles. This effect however was reduced when handling pre-treated samples (pH 1 and EDTA), nevertheless 1 month of preservation led to a 50% reduction of the original detected concentration.

Addition of HCl (pH 1) and EDTA proved to be most beneficial for the stability of low-level TU in urine of bovine and porcine origin. It resulted in a higher detection rate, translated not only in a higher number of samples positive for TU, but also in an increased concentration. Even more, a prolonged detection in time was observed. As for the storage conditions, the time spent at storage appeared responsible for the decrease in thiouracil concentration, rather than the number of freeze–thaw cycles, which is valuable information for transportation means (i.e. from sampling location to the analytical laboratory, etc.). These results imply that storage time should be kept to a minimum. For future purposes, it might be of interest to investigate the elimination kinetics of thyreostatic drugs in non-compliant urine samples, to establish a maximum duration on the storage time in case of confirmatory analysis.

Conclusion

The present study investigated the stability of thyreostatic drugs, comprising TAP, TU, BTU, MTU, MBI, PTU and PhTU in bovine as well as porcine urine. At room temperature, a linear decrease was observed in time for all analytes. When preserved at −70 °C on a short-term basis (4 days) this decrease was inversely related to the increase in number of freeze–thaw cycles. On a long-term basis (5 months) however, the number of freeze–thaw cycles proved irrelevant for the decrease in thyreostats concentration. In this case, the time of preservation was correlated with the concentration decrease. Additionally, an effective pre-treatment was developed by adjusting the pH value to 1 upon sampling and supplementing 0.1 mL of 0.1 M of EDTA. This treatment delayed the decrease in thyreostat concentration in urine, and even prolonged its detection over time. However for the incurred urine samples, this decrease or disappearance in signal for

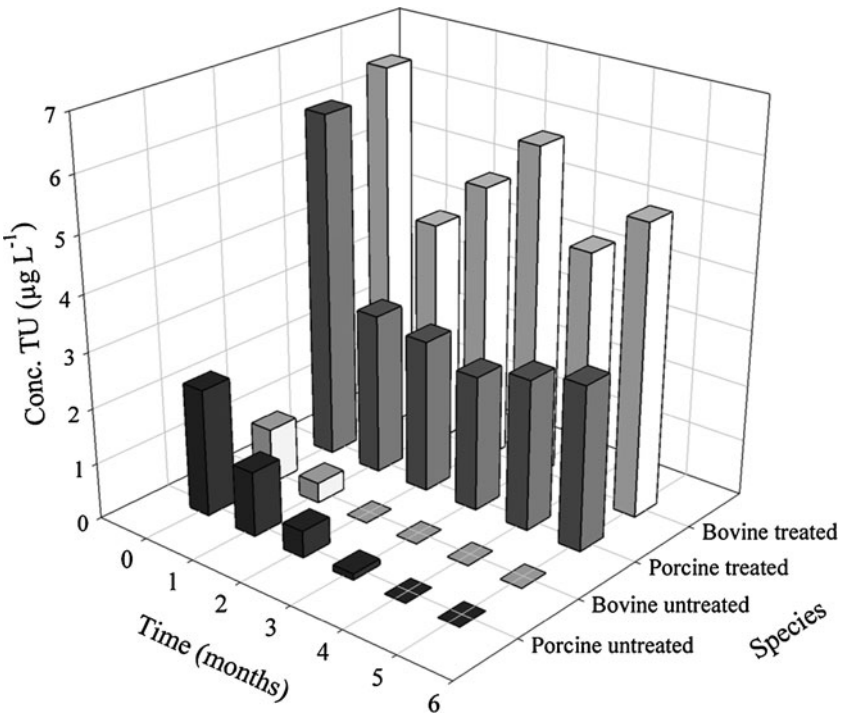

Fig. 4 The effect of pre-treatment (pH 1 and 0.1 mL of 0.1 M EDTA) on the stability of low-level thiouracil concentrations of incurred bovine (n=1) and porcine (n=1) urine samples during a 5 month preservation at −20 °C, with a monthly LC-MS^2 analysis

the low-level thyreostat (<10 μg L^{-1}), thiouracil was unavoidable with long-term preservation at −20 °C, even with pre-treatment. This is important knowledge for conducting residue analysis for legislative as well as research purposes. In the future, when analysing thyreostatic drugs in urine in light of national residue control plans, it is highly recommendable to conduct the suggested pre-treatment of pH 1 and EDTA upon sampling. Furthermore, it is advisable to limit the storage time of urine at room temperature, but also in freezer (−20 or −70 °C) to a maximum of 3 months, which should be a feasible period for the executing laboratories.

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