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Development and validation of an ultra-high performance liquid chromatography tandem mass spectrometry method for quantifying thyreostats in urine without derivatisation

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ABSTRACT

Thyreostatic drugs, illegally administrated to livestock for fattening purposes, are banned in the European Union since 1981 (Council Directive 81/602/EC). For monitoring their illegal use, sensitive and specific analytical methods are required. In this study an UHPLC-MS/MS method was described for quantitative analysis of eight thyreostatic drugs in urine, this without a derivatisation step. The sample pretreatment involved a reduction step with dithiothreitol under denaturating conditions at 65 °C, followed by liquid-liquid extraction with ethyl acetate. This analytical procedure was subsequently validated according to the EU criteria (2002/657/EC Decision), resulting in decision limits and detection capabilities ranging between 1.1 and 5.5 $\mu g\,L^{-1}$ and 1.7 and 7.5 $\mu g\,L^{-1}$, respectively. The method obtained for all, xenobiotic thyreostats, a precision (relative standard deviation) lower than 15.5%, and the linearity ranged between 0.982 and 0.999. The performance characteristics fulfill not only the requirements of the EU regarding the provisional minimum required performance limit (100 $\mu g\,L^{-1}$), but also the recommended concentration fixed at 10 $\mu g\,L^{-1}$ in urine set by the Community of Reference Laboratories. Future experiments applying this method should provide the answer to the alleged endogenous status of thiouracil.

1. Introduction

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 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 gastro-intestinal 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 $\mu g\,L^{-1}$ or $\mu g\,kg^{-1}$. In December 2007, the Community of Reference Laboratories (CRLs) posted a guidance paper containing their view on 'state of the art' analytical methods for the national residue control plan [8]. This document, which has no legal force (serves only as a technical guidance), comprises recommended concentrations of substances for which no maximum residue limits (MRLs) have been established [9]. The recommended concentration for analyzing thiouracil, methyl-thiouracil, propyl-thiouracil, and tapazole in urine and in the thyroid gland was set at a concentration of 10 $\mu g\,L^{-1}$.

The development of analytical methods, fulfilling the provisional MRPL or more recent the recommended concentration is challenging due to the noteworthy chemical properties of thyreostatic drugs. The polar, amphoteric character of these drugs, and their ability to adopt different tautomeric forms, negatively affects the extraction yield from biological samples, but also their chromatographic separation. Additionally, in case of mass spectromet-

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ric detection (MS) their small molecular weight (110–210 Da) limits the sensitivity. The majority of the currently available analytical methods circumvents these difficulties by applying derivatisation before analysis, which increases the molecular weight, lowers the polarity, and stabilizes the molecule in a single tautomeric form. For thin layer chromatography the use of derivatisation agent 7-chloro-4-nitrobenzo-2-oxa-1,3-diazol (NBD-Cl) has been reported [1,10–13], whereas for gas chromatography coupled to MS, benzylchloride [14], methylating agents [15–17] or MSTFA (N-methyl-N-trimethylsilyl-trifluoroacetamide) have been used. The extraction yield prior to GC–MS analysis could be even more increased when combined with other derivatisation agents, specifically used for stabilizing the compounds in one tautomeric form, e.g. pentafluorobenzylbromide (PFBBr) [18–21] and 3-bromobenzylbromide (3-BrBBR) [21,22]. For LC-MS analysis only the use of NBD-Cl [23] and more recently 3-iodobenzylbromide [24,25] have been described. A more detailed overview of the substantial evolution in thyreostatic analysis has been reviewed earlier [26].

For routine analysis, the method of Pinel et al. [24], exploiting a 3-iodobenzylbromide derivatisation is generally accepted as the most optimal procedure [26,27]. This method generated decision limits (CC_α) and detection capabilities (CC_β) in the range of 0.1–5.2 and 2.6–23.2 $\mu g\,L^{-1}$, respectively. Recently Lõhmus et al. [25] transferred this method to the new ultra-performance liquid chromatographic (UPLC) technique coupled to tandem MS and obtained for all thyreostats CC_α and CC_β values in agreement with the CRL guidance paper (<10 $\mu g\,L^{-1}$). Application of these highly sensitive analytical procedures as opposed to earlier methods for urine of cattle, gave rise to the detection of thiouracil (TU) in the concentration range of 0–10 $\mu g\,L^{-1}$. The origin of this signal, which is assumed to be illegal administration, is however still a matter of debate since this signal could be retrieved in urine of untreated cattle as well [21,28]. Identification was carried out by 3 independent mass spectrometric approaches (i.e. LC–MS/MS, GC–MS/MS and HRMS), each using a different derivatisation procedure [21]. These approaches acknowledged the presence of TU in urine of untreated cattle. For this reason Pinel et al. [28] proposed the possible endogenous formation of the xenobiotic thyreostat, thiouracil. However, the liability of a derivatisation step, which may result in possible false-positive identification of TU should be considered. For investigation of this suggested endogenous status, the development of a highly sensitive method for the detection of underivatised thyreostats, more specific for thiouracil in urine could offer a conclusive answer. In addition, the avoidance of a derivatisation step simplifies the sample pretreatment and reduces laboratory costs. Recently Abuin et al. [29,30] published such a simplified method, in thyroid tissue. Nevertheless, this method for underivatised thyreostats obtained too high values for the decision limit and detection capability.

Therefore, the aim of the present study was to develop a confirmatory quantitative method for the determination of underivatised thiouracil in urine aliquots, which for future reference could be employed to conclude on the paradox surrounding the semi-endogenous status of TU. To this purpose ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry (UHPLC-MS/MS) was used. Subsequently, the method was validated according to the criteria set in Commission Decision 2002/657/EC [31]. All xenobiotic thyreostats, monitored by the European control plans were included in the validation.

2. Materials and methods

2.1. Reagents and chemicals

Standards were obtained from Sigma–Aldrich (St. Louis, MO, USA). Stock solutions of the thyreostatic drugs: 2-thiouracil (TU), 6-dimethyl-thiouracil (DMTU), 6-ethyl-thiouracil (ETU), 6-methyl-2-thiouracil (MTU), 6-propyl-2-thiouracil (PTU), 6-phenyl-thiouracil (PhTU), 1-methyl-2-mercaptoimidazole (tapazole, TAP) and 2-mercaptobenzimidazole (MBI) were prepared in methanol at a concentration of 200 ng μL^{-1}. Working solution was prepared by 200× and 2000× 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 in the refrigerator.

The deuterated internal standard (IS), PTU-D5 was provided by Toronto Research Chemicals Inc. (Toronto, Canada).

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

Phosphate buffer, dissolved in deionized water, was controlled and adjusted to a pH of 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).

2.2. Instrumentation

The LC system consisted of a Thermo Electron (San José, USA) Accela UHPLC pumping system, coupled with an Accela Autosampler and Degasser. Chromatographic separation was achieved by reversed phase chromatography and gradient elution. Separation of the thyreostatic compounds was carried out on a Acquity UPLC HSS T3 column (High Strength Silica particles) (1.8 μm, 100 mm × 2.1 mm, Waters, Milford, MA, USA), kept at 35 °C. An Acquity UPLC in-line filter (2.1 mm, 0.2 μm, Waters) was used to improve analytical column lifetime. The mobile phase constituted 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}. Optimized separation of all 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. All analytes could be separated in a total runtime of only 9.4 min. Analysis was performed on a triple quadrupole mass analyzer (TSQ Vantage, Thermo Electron, San José, USA), fitted with a heated electrospray ionization (HESI II) source operating in positive ion mode. The following working conditions were applied: spray voltage at 3.5 kV; vaporizer and capillary temperature at 370 and 300 °C, respectively; sheath and auxiliary gas at 40 and 20 arbitrary units (a.u.), respectively; 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 optimized individually per compound (Table 1). Quantification and confirmation data were acquired in selected reaction monitoring (SRM) mode, the transitions followed are displayed in Table 1. Instrument control and data processing were carried out by means of Xcalibur Software (Thermo Electron, San José, USA).

2.3. Samples

Urine samples from bovine, ovine, and porcine origin were obtained from veterinary sampling in light of the European residue control plan. Upon arrival at our laboratory, samples were stored at −20 °C, and thawed before analysis.

The thawed samples were centrifuged for 10 min at 4000 × *g*, and aliquots of 1 mL were used for analysis. To each sample 50 ng of internal standard (PTU-D5) was added, to obtain a final concen-

Table 1
Collected SRM transitions and compound specific MS parameters (product ions in bold were used for quantification purposes).

Analyte	tR (min)	Precursor ion (*m*/*z*)	Product ions (*m*/*z*)	S-lens (RF amplitude) (V)	Collision energy (eV)
TU	1.64	129.0	**112.1**	49	15
			84.1		27
			60.1		34
			57.1		37
TAP	2.28	115.0	81.1	54	32
			74.1		17
			57.2		19
MTU	2.93	143.0	**126.1**	53	15
			86.1		23
			84.1		17
			60.1		36
DMTU	4.56	157.1	**140.1**	67	17
			98.2		19
			72.1		34
			60.1		37
ETU	4.77	157.1	78.2	48	23
			72.1		36
			60.1		36
PTU	5.44	171.1	**154.1**	60	16
			122.2		18
			86.1		25
			60.1		36
PTU-D5[a]	5.42	176.1	**159.2**	62	17
			117.2		19
			86.1		28
			60.1		34
MBI	5.33	151.0	**118.1**	67	25
			93.1		23
			91.1		34
			65.2		36
PhTU	6.04	205.0	**188.1**	102	18
			146.1		19
			103.1		27
			77.1		38

[a] Internal standard (IS).

tration of 50 μg L^{-1}. As for the spiked samples, a standard solution (0.1 or 1.0 ng μL^{-1}) containing all 8 thyreostats was added, vortexed vigorously for 1 min and left to equilibrate for at least 15 min before extraction.

2.4. Sample extraction and purification

One mL of DTT-enriched (1%) phosphate buffered saline at pH 7 was added to each sample, followed by vortexing vigorously for 1 min. Subsequently, the samples were placed under denaturating conditions, at 65 °C for 30 min, next the denaturated, reduced extracts were purified with 2× 5 mL ethyl acetate. Finally, the combined extracts were evaporated to dryness under a gentle stream of nitrogen at 60 °C and the dried residue redissolved in a total volume of 200 μL of mobile phase consisting of 10/90 MeOH/0.1% aqueous formic acid. This ratio is slightly different than the initial conditions of the UHPLC-MS/MS procedure, but the elevated methanol concentration was necessary to ensure good dissolvation of the analytes. Of the obtained extract, 10 μL was injected on column.

2.5. Quality assurance

Prior to the sample analysis, a standard mixture of the targeted 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 50 μg L^{-1}, prior to the extraction. The identification of the thyreostats were based on their 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 [31]. After identification, the analytes concentration was calculated by fitting its area ratio in a eight-point calibration curve, established by blank urine samples spiked with 8 thyreostats in the range of 2.5–100 μg L^{-1} and the IS at 50 μg L^{-1}.

Area ratios were determined by integration of the area of an analyte under the specific SRM chromatograms in reference to the integrated area of the internal standard.

3. Results and discussion

3.1. UHPLC and MS parameters

Initially, our analytical procedure was developed on a HPLC system (Finnigan Surveyor, Thermo Electron, San Jose, CA, USA) coupled to a LTQ linear ion trap mass analyzer (Thermo Electron, San Jose, CA, USA). To determine optimal MS conditions, the default parameters for the HESI-probe, capillary temperature, vaporizing temperature, sheath gas pressure, and auxiliary gas pressure were further optimized by individually infusing the analytical thyreostatic standards. During this tuning step acetic acid and formic acid were evaluated as candidate mobile phase additives, to enhance ionization. Addition of formic acid at 0.1% in the mobile phase provided the most optimal compromise between ionization and peak geometry. For chromatographic separation different columns, with a special emphasis on columns with a higher affinity for polar compounds were tested, i.e.

normal reversed phase Symmetry C18 (5 μm, 150 mm × 2.1 mm, Waters), Cogent Bidentate C18 (4 μm, 75 mm × 2.1 mm, Micro Solv), Gemini C6-phenyl (3 μm, 50 mm × 2.0 mm, Phenomenex), Nucleodur Sphinx (5 μm, 250 mm × 4.0 mm, Machery-Nagel), Atlantis T3 (3 μm, 150 mm × 2.1 mm, Waters), and Hypercarb (5 μm, 100 mm × 2.1 mm, Thermo Scientific). The choice of column was mainly determined by the achieved retention time, the base peak separation, and the peak efficiency, measured as peak width at the baseline. Evaluation of the retention was based on the earliest eluting analyte thiouracil. Reasonable retention times were obtained with the Nucleodur Sphinx, Atlantis T3, and Hypercarb column and these were subjected to further investigations. Subsequently, base peak separation of structurally related thyreostats (ETU and DMTU) was compared, for which all three columns obtained good results. With regard to the peak efficiency, the Hypercarb displayed relatively broad peaks, whereas the Altantis T3 provided the narrowest peaks. This could be explained by the relative small particle size of the column, 3 instead of 5 μm. Therefore, the Atlantis T3 column was chosen for chromatographic separation of thyreostatic drugs. Additionally, retention times, optimal separation, and good resolution were ameliorated by careful selection of the gradient program. Moreover the methanol gradient was chosen in such a manner that reasonable retention times and a good base peak separation of structurally related thyreostats occurred. The organic solvent methanol was preferred, as the higher elution strength of acetonitrile proved to be disadvantageous for retention of the relatively polar thyreostats.

According to Commission Decision 2002/657/EC [31], LC–MS/MS analysis of banned substances requires 4 identification points (IP), which accords to one precursor (1 IP) and two product ions (2 × 1.5 IP). However, upon fragmentation with the linear ion trap mass analyzer only 1 product ion could be observed. Therefore the use of a triple quadrupole analyzer (QqQ) was preferred. Since this high-end fast-scanning QqQ apparatus allows the combination with ultra-high performance liquid chromatography, a method transfer to UHPLC was performed at the same time. The equivalent U(H)PLC columns: Nucleodur Sphinx (Machery-Nagel), Acquity HSS T3 (Waters), and Hypersil Gold (Thermo Scientific) of those providing the best retention and base peak separation in classical HPLC were evaluated for their performances. The Acquity HSS T3 (1.8 μm, 50 mm × 2.1 mm) provided not only the highest signal to noise ratio (S/N) and peak intensity, but most important the best retention of TU. When analyzing pre-spiked urine extracts, detection of thiouracil was ambiguous. Subsequently, some of these analyzed extracts were post-spiked to investigate the ion suppression phenomenon [32]. Post-spiking resulted in the detection of TU, linking the lack in detection to co-elution of isobaric interferences. This problem was resolved when switching to a longer, 100 mm instead of 50 mm HSS T3 column, as depicted in Fig. 1. Indeed, Wren [33] acknowledges that the use of longer columns results in better resolution because of greater retention and selectivity.

The protonated molecular ion $[M+H]^+$ was selected as precursor ion for all compounds, four transitions per analyte were acquired, except for TAP and ETU for which only 3 transitions could be obtained (Table 1). For each compound, based on peak intensity and signal to noise ratio, the two most intense transitions were selected for validation purposes.

3.2. Development of sample pretreatment

Preliminary experiments were performed to evaluate the performance of different solvents in extracting thyreostats form urine. Therefore three organic solvents, immiscible with water but polar enough to extract thyreostats, were selected, i.e. chloroform, dichloromethane, and ethyl acetate. Before extracting fortified urine samples, experiments with fortified water samples were conducted during which ethyl acetate obtained the highest extraction yield. However when applied to urine samples, even with optimization of the pH (pH 2–10) no thyreostatic drugs could be detected. As a result of these observations, the influence of a clean-up step prior to the analysis of underivatised thyreostats was investigated. In literature the use of Silica solid phase extraction (SPE) cartridges has been described for thyreostats [23,29,34]. This SPE sorbent was applied in an indirect procedure, LLE prior to Si SPE, as well as in a direct one, redissolving an evaporated urine sample in a less polar, more desired solvent before loading onto the sorbent. Other types of SPE sorbents were also tested, e.g. Oasis HLB/WAX/MAX (Waters), Envi-Carb (Supelclean), and HR-X (Machery-Nagel), but all unsuccessful when applied to urine samples. Some of these samples were post-spiked upon detection to check for ion suppression, but this was not the case. Therefore, the hypothesis was posed that endogenous matrix constituents present in urine interfered with the extraction. Most likely this concerns some kind of protein as indicated by Blanchflower et al. [34].

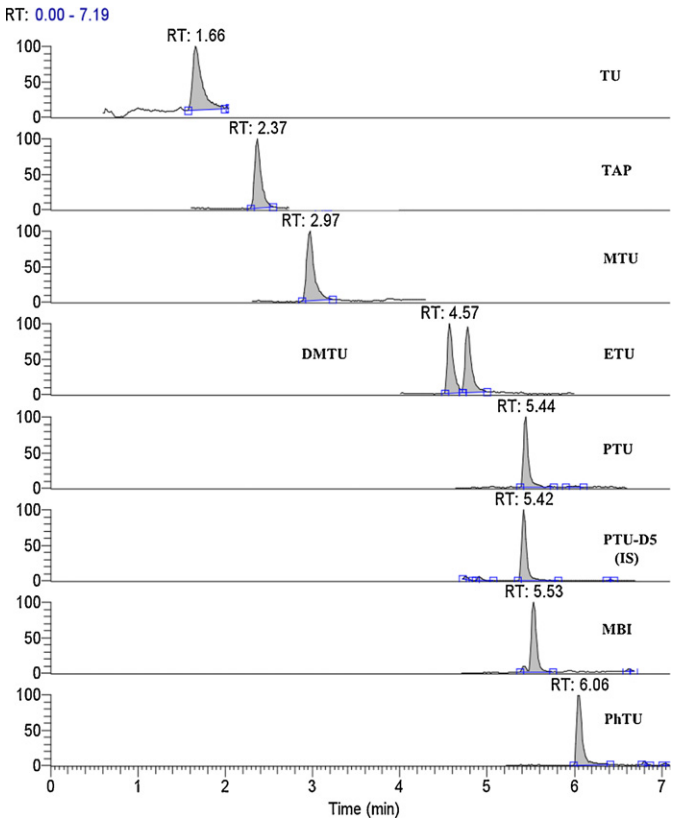

Fig. 1. SRM chromatogram of an extracted urine, spiked with 8 thyreostats at 10 μg L^{-1} and the internal standard, PTU-D5 at 50 μg L^{-1}.

Therefore, different strategies to eliminate these interferences and allowing the extraction of thyreostats from urine were compared. During a first series of experiments, the known interaction between thyreostats and metal ions was exploited [1,13,35,36]. To this purpose, metal ions, more specific Mn^{2+} was added to the sample in order to interact with the thiol group of thyreostatic drugs and enhance the extraction yield [35]. An alternative strategy was preventing these interactions by adding the chelating agent ethylenediaminetetraacetate (EDTA) [27,34]. Salting out (NaCl; 2.5–5 g) to aid the partition of the thyreostats into organic solvent was also tested, as was potential hydrolysis with β-glucuronidase (50–100–150–200 μL; 2–4 h-overnight; 50–60–65 °C). A second series of experiments focused on the potential disruption of protein–thyreostat interaction, and more

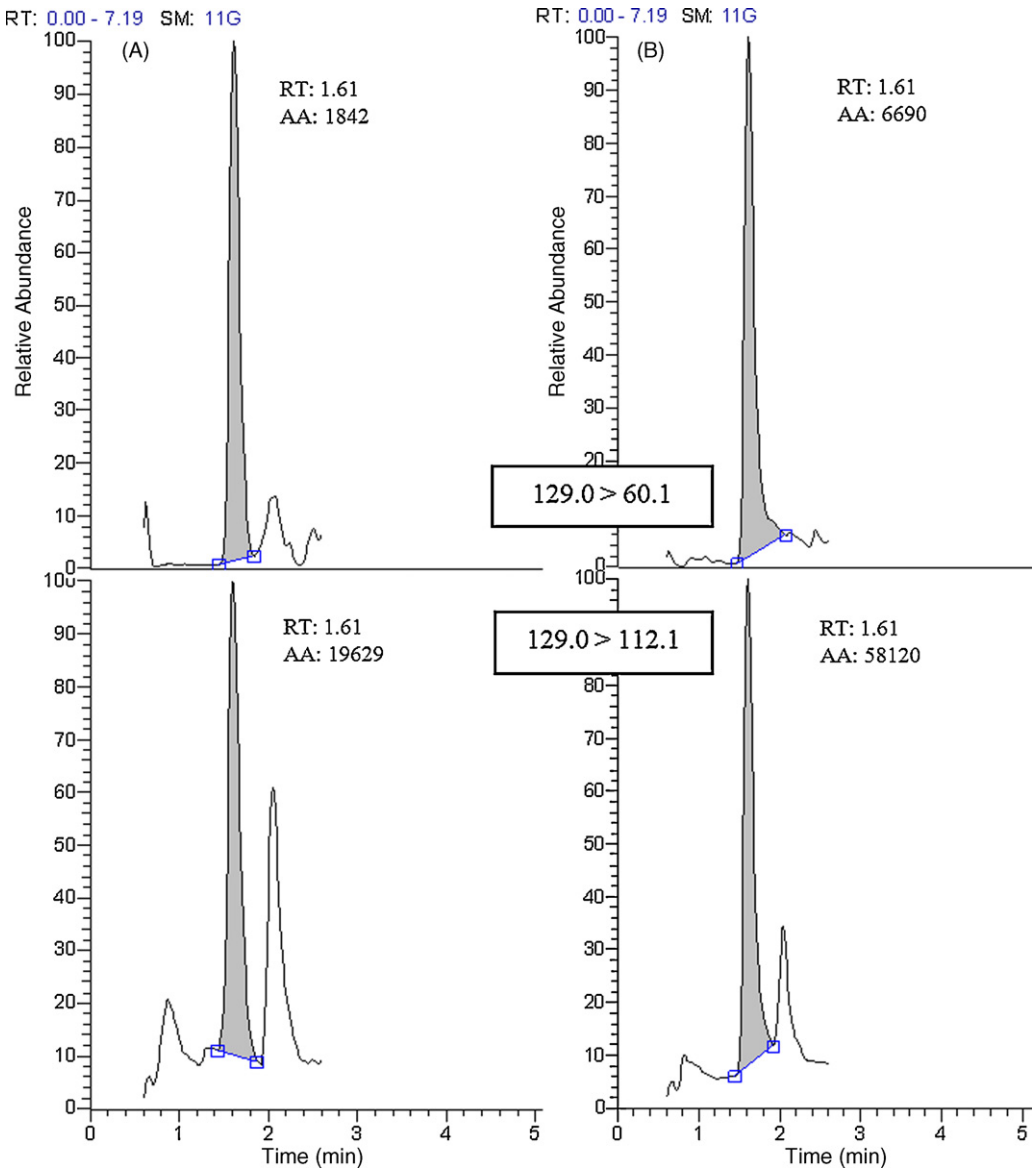

Fig. 2. SRM chromatogram of a blank urine sample (mixture of 6 different urines) fortified at 10 μg L^{-1} of thiouracil, where zirconia beads (A) and DTT (B) were used to aid in the extraction.

specific denaturation of matrix proteins. To this end, the supplementation of different organic solvents, i.e. acetone, methanol, acetonitrile (0.5–1.0–2.0–3.0 mL), and an acidic treatment (HCl; 1–6 M; 50–100–200 μL) were exploited. Subsequently, the impact of mechanical disruption was evaluated by adding zirconia beads (Ø 0.1–0.5 mm, 0.5–0.75–1.0 g) at elevated temperatures (60–65 °C; 2–4 h) [37,38]. Finally, the supplementation of reducing agent DTT or the anionic surfactant SDS (sodium dodecyl sulfate) (1 mL of buffer saturated with 1%), as a means to disrupt disulfide or non-covalent bonds, respectively, was examined [39,40].

The two most promising methods were mechanical disruption using zirconia beads and addition of the reducing agent DTT. The beads application has been reported for disrupting bacterial, yeast, and fungal cells to extract DNA [37,38,41,42], whereas DTT has been for cleaving disulfide bonds [39]. Both protocols were subjected to further experimentation. Parameters such as the size and amount of the beads, pH, temperature, and means of disruption were important for optimization of the mechanical disruption procedure. In the finalized protocol 1 g of zirconia beads (Ø 0.5 mm) was supplemented to the urine sample set at pH 5.2, afterwards placed for 2 h in an ultrasonic bath at 65 °C, with additional manual shaking, followed by LLE. For the sample pretreatment protocol applying DTT, the pH of the sample proved to be of crucial importance, since the reducing power of DTT is limited to pH values above 7. However, the addition of DTT (1 mL of buffer at pH 7, 1% DTT) alone proved to be insufficient, since no extraction of thyreostats occurred. This indicated that the disulfide bonds were buried, and thus not accessible to solvents. Therefore the protocol had to be carried out under denaturating conditions, 65 °C, which resulted in the extraction of thyreostats from urine. The extraction yield was not improved by increasing the duration of the denaturation step and therefore kept at 30 min. When comparing the two finalized protocols for zirconia beads and DTT ($n = 18$), normalized area ratios of respectively, 0.0039 ± 0.006 (CV 15.9%) and 0.054 ± 0.007 (CV 12.8%) were obtained. In the end, the DTT procedure proved to be the most sensitive method, as demonstrated in Fig. 2, but also the least liable to differences in urine samples, and therefore selected for validation purposes.

3.3. Validation

The newly developed analytical method was validated according to the criteria specified in Commission Decision 2002/657/EC for quantitative confirmation [31]. For each compound 4 transitions were monitored, except for TAP and ETU (see Table 1). The primary and secondary product ions were used for quantification purposes.

Firstly, an appropriate internal standard was chosen, capable of anticipating fluctuations in the signal intensity upon extraction of thyreostats from urine samples. The use of an isotopically labeled IS in MS-based chemical analysis has always been recommendable, as well as compounds that are structurally related to the analyte (basic structure identical) [31]. For these reason the deuterated propylthiouracil (PTU-D5), DMTU, and ETU were compared as internal standards. Due to its superior performance PTU-D5 was preferred and used as IS throughout this study.

The validation procedure used, adopted in part a particular protocol proposed by Antignac et al. [43]. This was tailored for validating analytical methods based on MS detection and tried to give a compromise between the 2002/657/EC European Decision requirements and practical aspects and limitations related to laboratory work [31]. The validation protocol was designed as follows.

Analysis of 20 blank urine samples from various origins (bovine, porcine, and ovine) was performed to check for the ruggedness of the method. This set up permitted to determine the specificity by calculating the average (μ_N) and standard deviation (σ_N) of the noise amplitude, expressed relative to the internal standard signal amplitude. The calibration curve was realized on a mixture of 6 previously analyzed blank urine samples. Seven fortification levels were included with the previously estimated noise average (μ_N) as a forced intercept. The linearity of this calibration graph was evaluated by calculation of the correlation coefficient (R^2) and the sensitivity, i.e. the slope of the fitted curve (a). Based on these data, the decision limit (CC_α) was calculated taking in account the equation of the calibration graph (Eq. (1)), where I is the signal amplitude and C the concentration, and the definition of CC_α (Eq. (2)). This combination led to the expression given by Eq. (3):

$$I_{CC_\alpha} = \mu_N + aCC_\alpha \quad (1)$$

$$I_{CC_\alpha} = \mu_N + 2.33\sigma_N \quad (2)$$

$$CC_\alpha = \frac{2.33\sigma_N}{a} \quad (3)$$

For calculating CC_β, 20 blank samples were spiked at the determined CC_α level. This permitted to estimate the repeatability through the standard deviation of the signal amplitude (σ_S). In order to minimize the estimation error, the signal relative standard deviation ($(RSD)_S$) was preferred above the standard deviation (σ_S). Finally with σ_N, a, and $(RSD)_S$, the detection capability (CC_β) could be calculated, taking in account the calibration equation (Eq. (4)), where I is the signal amplitude and C the concentration, and the definition of CC_β (Eq. (5)). The combination of these two formula led to the final expression given by Eq. (6):

$$I_{CC_\beta} = \mu_N + aCC_\beta \quad (4)$$

$$I_{CC_\beta} = \mu_N + 2.33\sigma_N + 1.64(RSD)_S I_{CC_\beta} \quad (5)$$

$$CC_\beta = \frac{2.33\sigma_N + 1.64\mu_N(RSD)_S}{a[1 - 1.64(RSD)_S]} \quad (6)$$

For the mean recovery and precision, 18 identical blanks, originating from a mixture of 6 different urines were amended with targeted compounds at 0.5, 1.0, and 1.5 times the recommended concentration of 10 $\mu g\,L^{-1}$ and the IS, splitted in three sets ($n = 6$). This was performed by two operators on three different days.

3.3.1. Specificity

The specificity of the method was demonstrated by analysis of blank urine samples and samples fortified with each analyte separately or with a mixture of all analytes. The fortification level was set at 10 $\mu g\,L^{-1}$, in accordance with the recommended concentration of the CRL guidance document (2007) [8]. More recently, endogenous production of TU in the 0–10 $\mu g\,L^{-1}$ range has been reported [28,25]. Therefore blank urine samples ($n = 20$), containing a background concentration lower than 1 $\mu g\,L^{-1}$ of TU were selected to validate the method. For each analyte spiked, chromatograms showed a significant increase in peak area and intensity at its specific retention time compared to the blanks, taking a signal to noise ratio of at least 3 into account (Fig. 1). No other matrix substances interfered at these retention times. Therefore, the newly developed method was found to be specific for all eight thyreostatic drugs (TU, TAP, MTU, DMTU, ETU, PTU, MBI, and PhTU) in the presence of matrix components.

3.3.2. Selectivity

In accordance with the European Criteria 2002/657, analytes were identified on the basis of their relative retention time, i.e. the ratio of the chromatographic retention time of the analyte to that of the internal standard [31]. In addition, a system of identification points was used to interpret the data, based on the ion ratios of the precursor and product ions in the acquired spectrum [31]. For the confirmation of thyreostats, listed in Group A of Annex I of Directive 96/23/EC [44], a minimum of 4 identification points (IPs) is required [31]. Precursor (1 IP) and product ions (1.5 IP/ion) of each analyte are presented in Table 1. The individual relative retention time ($n = 18$) of the extracted thyreostatic compounds showed in every case a standard deviation lower than 0.006, with a coefficient of variation smaller than 0.90%. This falls well within the stated tolerance level of 2.5% for liquid chromatography. As a result, the identification of the eight thyreostatic compounds, extracted from urine samples was unambiguously. As for the identification points, a maximum of 7 could be designated to the analytes with 4 monitored transitions. As for TAP and ETU, where only three transitions were monitored, a maximum of 5.5 IP's could be assigned. When analyzing urine samples fortified at 5–10–15 $\mu g\,L^{-1}$, the minimum required amount of IP's, set at 4, was easily achieved.

3.3.3. Calibration curves

The linearity of the developed method was evaluated for each thyreostatic compound by preparing calibration curves in blank matrix, which consisted of a mixture of 6 different bovine urines. The blank samples were fortified within a range of 2.5–100 $\mu g\,L^{-1}$, but no equidistant steps were used. More notice was given to the low concentration range, since this part strongly influences the CC_α and CC_β values. Moreover by using these fortification levels, the concentration domain globally used in practice (0–100 $\mu g\,L^{-1}$) could be verified. Linear regression analysis was carried out by plotting the peak area ratios of the analyte against the IS versus the analyte concentrations. The estimated noise average of the pool of blanks ($n = 20$) was used as a forced intercept [43]. Good linearity was obtained, all correlation coefficients (R^2) were $\geq$0.991, only TAP was slightly inferior, but still acceptable at 0.982.

3.3.4. Mean recovery

Since no certified reference material was available, trueness of the measurements was assessed by fortifying blank urine samples (pool of 6) with 0.5, 1.0, and 1.5 times the recommended concentration as stated in the CRL guidance document (10 $\mu g\,L^{-1}$). This was performed in six replicates. As can be deduced from Table 2, all calculated mean recoveries fulfill the criteria put forward in the EC/2002/657 stating that a mass fraction between 1 and 10 $\mu g\,L^{-1}$

Table 2
Precision and mean recovery of the developed method for eight thyreostats, analyzed in urine.

Analyte	Nominal concentration ($\mu g\,L^{-1}$)	Recovery[a]		Repeatability[b]		Within-laboratory reproducibility[c]
		Mean ± SD (%)	RSD (%)	Overall mean ± SD ($\mu g\,L^{-1}$)	RSD (%)	RSD (%)
TU	5	91.2 ± 16.1	17.6			17.7
	10	89.1 ± 12.4	14.0	9.8 ± 1.4	14.0	12.3
	15	98.2 ± 10.4	10.6			7.5
TAP	5	103 ± 16.2	15.8			16.4
	10	101 ± 14.6	14.4	11.3 ± 11.9	11.9	13.5
	15	105 ± 11.2	10.7			
MTU	5	106 ± 4.8	4.5			5.5
	10	103 ± 2.5	2.5	10.4 ± 0.8	7.4	4.8
	15	91.1 ± 2.3	2.5			9.2
DMTU	5	92.8 ± 14.9	16.0			18.0
	10	111 ± 13.2	11.9	10.8 ± 1.6	14.5	13.1
	15	110 ± 10.1	9.2			8.8
ETU	5	91.5 ± 10.6	11.5			12.2
	10	107 ± 13.1	12.2	10.3 ± 1.2	12.1	9.9
	15	109 ± 11.9	10.9			12.1
PTU	5	95.1 ± 4.6	4.9			6.5
	10	100 ± 1.7	1.7	10.5 ± 0.7	6.2	6.0
	15	104 ± 3.7	3.5			6.2
MBI	5	99.5 ± 10.1	10.1			12.2
	10	105 ± 13.0	12.3	10.5 ± 0.9	8.9	10.4
	15	107 ± 10.3	9.6			11.9
PhTU	5	93.3 ± 11.9	12.8			16.7
	10	108 ± 15.4	14.3	11.0 ± 1.8	16.4	12.2
	15	110 ± 11.9	10.9			15.4

[a] 18 aliquots of identical blank samples, and fortify six aliquots at each of 0.5, 1.0, and 1.5 times the recommended concentration of 10 $\mu g\,L^{-1}$.
[b] Three series of six replicates of fortified samples of an identical matrix at 0.5, 1.0, and 1.5 times the recommended concentration of 10 $\mu g\,L^{-1}$, under identical conditions.
[c] Four series of six replicates of fortified samples of an identical matrix at 0.5, 1.0, and 1.5 times the recommended concentration of 10 $\mu g\,L^{-1}$, analyzed by two different operators.

should obtain a mean recovery range of 70–80%, whereas a mean recovery of 80–110% should be required for a mass fraction of, or greater than 10 $\mu g\,L^{-1}$. Only DMTU, which exceeds this limit by 1% at 1 time the fortification level, did not fulfill this criterion. The recoveries (98.1–111%) obtained with this innovative analytical method were highly satisfactory. However to the best of our knowledge, besides an older study of Blanchflower et al., which obtained inferior recoveries, little has been reported on the recoveries of thyreostatic drugs in urine [34]. More recent reports concerning urine did not mention recoveries [24,25], or handled extraction of other matrix like the thyroid gland with recoveries lower than 75% [18,45].

3.3.5. Precision

To evaluate the precision of the method, repeatability and within-laboratory reproducibility were determined. Repeatability was evaluated by calculating the coefficients of variation (CV). To this purpose, data from three series of six replicates of samples of an identical origin fortified at 0.5, 1.0, and 1.5 times the recommended concentration of 10 $\mu g\,L^{-1}$ were used. These analyses were carried out on different occasions by the same analyst under repeatable conditions. For all thyreostats considered, good repeatability was obtained, since the individual overall calculated CVs for each compound were well below 20% (Table 2). This outcome was not evaluated by the Horwitz equation, too high values would be obtained, because of the low concentration range used [31]. However, in accordance with the Commission Decision 2002/657/EC, the CVs obtained for mass fraction lower than 100 $\mu g\,L^{-1}$ were as low as possible [31].

For evaluation of the reproducibility only the within-laboratory reproducibility was considered. Four series of six replicates of fortified samples at 0.5, 1.0, and 1.5 times the recommended concentration of 10 $\mu g\,L^{-1}$ were analyzed by different operators on different days. The results, summarized in Table 2 indicate the good precision of the method. The obtained CVs were in accordance with the commission Decision (2002/657/EC) stating that in case of repeated analysis of a sample carried out under within-laboratory reproducibility conditions, the intra-laboratory coefficient of variation of the mean should not exceed 20% in case of a mass fraction of, or greater than 10 $\mu g\,L^{-1}$.

3.3.6. Decision limit (CC_α) and detection capability (CC_β)

Different procedures to determine the decision limit (CC_α) and the detection capability (CC_β) are reported in literature [31,43]. The decision limit CC_α is defined as the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant. During this study, the CC_α and CC_β were determined by analysis of 20 blank urine samples, respectively, non-fortified and fortified at CC_α level. The signal associated with CC_α corresponds to the maximal noise amplitude. The detection

Table 3
Decision limits (CC_α) and detection capabilities (CC_β) calculated for the eight thyreostats in urine according to the 2002/657/EC Decision.

Analyte	CC_α ($\mu g\,L^{-1}$)	CC_β ($\mu g\,L^{-1}$)
TU	2.2	3.0
DMTU	1.7	2.3
ETU	1.2	2.1
MTU	1.1	1.7
PTU	2.2	3.3
PhTU	1.6	2.4
TAP	5.5	7.5
MBI	1.1	1.7

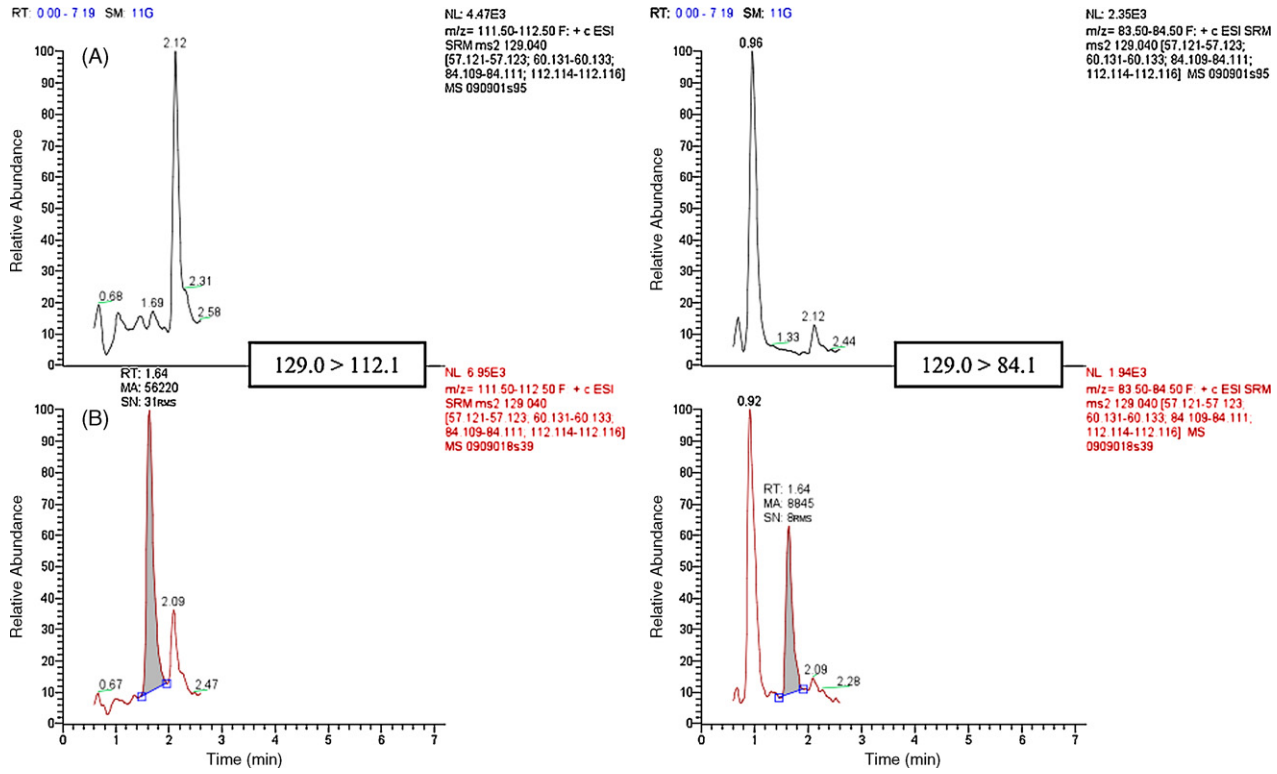

Fig. 3. SRM chromatogram of a blank urine sample reduced by DTT and subsequently extracted with ethyl acetate (A), and a fortified urine at the CC_α level of thiouracil ($2.2\ \mu g\ L^{-1}$) (B).

capability CC_β is defined as the lowest concentration at which a method is able to detect contaminated samples with a statistical certainty of $1-\beta$ (error probability = 5%). Table 3 summarizes the calculated CC_α and CC_β values for the different thyreostats. Because the concentrations for CC_α, obtained by this approach concern relatively low values, a preliminary experiment was conducted to check if al compounds were detected when spiked at their CC_α level (Fig. 3). The determination of CC_α and CC_β occurred in the context of a confirmatory method for banned substances, as such a minimum of 2 product ions was required. Subsequently, the two most intense transitions were used for the calculations. Decision limits and detection capabilities ranged, respectively, between 1.1 and 5.5 $\mu g\ L^{-1}$ and between 1.7 and 7.5 $\mu g\ L^{-1}$. These results are highly satisfactory since the performances are far below the suggested minimum required performance limit of 100 $\mu g\ L^{-1}$ in urine samples (MRPL). Even so, if in the future the recommended concentration (CRL guidance document 2007) [8] of 10 $\mu g\ L^{-1}$ for TU, MTU, PTU, and TAP in urine would be legalized, this method would still easily meet the requirements.

4. Conclusion

Ultra-high performance liquid chromatography coupled to tandem mass spectrometry proved to be a suitable technique for the quantification and confirmation of eight thyreostats (TU, TAP, MTU, DMTU, ETU, PTU, MBI, and PhTU) in urine samples without derivatisation. The selected reaction monitoring mode of the triple quadrupole mass analyzer easily allowed quantification at the level of the recommended concentration of 10 $\mu g\ L^{-1}$ for TU, MTU, PTU and TAP, resulting in low CC_α and CC_β values. Moreover, the newly developed UHPLC protocol enabled shorter analysis times (10 min) and, consequently, a higher throughput, while maintaining good peak separation and resolution. The concise sample pretreatment, consisting of a reduction step followed by a simple LLE even further allowed to reduce analysis time and costs. Omitting the derivatisation resulted not only in a decreased analysis time and laboratory costs, but should also reduce the possibility of obtaining possible false-positive results. To that purpose, the application of this method in laboratories involved in the official control of residues of thyreostatic drugs in urine samples could be highly advantageous.

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