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Control of residues of thyreostats in slaughter animals in Poland in 2011–2017

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Abstract

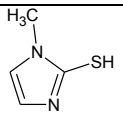
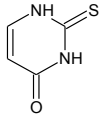
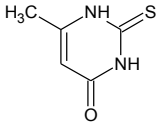
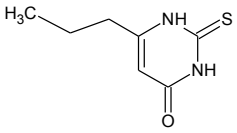
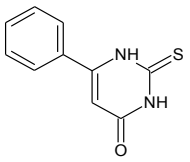
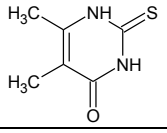
Introduction: In the European Union, the use of thyreostatic drugs for fattening slaughter animals has been banned since 1981 under Council Directive 81/602/EEC. For protection of consumer health against unwanted residues and in compliance with Directive 96/23, each EU country must monitor thyreostats in samples of animal origin. This paper presents the results of research on thyreostatic residues carried out in Poland in 2011–2017. **Material and Methods:** The material for testing was urine (n = 3,491), drinking water (n = 127), and muscle samples (n = 349) officially collected by Veterinary Sanitary Inspectors in slaughterhouses and farms throughout the country in accordance with the national residue control plan. The samples were examined for the presence of tapazole, thiouracil, methylthiouracil, propylthiouracil, and phenylthiouracil using liquid chromatography tandem mass spectrometry through an accredited method. **Results:** In four bovine and three porcine urine samples, the permissible thiouracil concentration was exceeded. In one sample of porcine urine, methyl- and propylthiouracil were found. The presence of thiouracil and its derivatives in urine samples is most likely due to feeding animals diet containing cruciferous plants. **Conclusions**: The results of research indicate that thyreostats are not used for anabolic purposes in slaughter animals in Poland.

Keywords: thyreostats, thiouracil, urine, monitoring.

Introduction

Thyreostatic substances, also called antithyroid agents, affect the functioning of the thyroid glands through the blockade of thyroid hormone synthesis (2). The hormones produced by the thyroid gland are triiodothyronine (T3) and thyroxine (T4), and their deficiency favours the animal's fattening because both hormones are involved in metabolism regulation. An increase in animal body weight is the result of water retention in subcutaneous and muscular tissues, as well as in the gastrointestinal tract (22). The use of thyreostatic substances in breeding for fattening causes relatively rapid animal body weight gain. These compounds were recognised and consciously exploited by cattle farmers in the 1980s in some European countries. The more, that thyreostats are easy to use, because they are active per os and effective after administration with feed. Due to the toxicity, carcinogenic and teratogenic properties of thyreostats (10), their use in the production of food of animal origin has been forbidden in the European Union since 1981 (5, 6). Prohibition also means that thyreostatic residues should not be present in animal tissues; therefore, there are no maximum residue limits for this group of compounds. The best known antithyroid agents used to intensify fattening of animals include methylthiouracil (MTU), tapazole (TAP), thiouracil (TU), propylthiouracil (PTU), and phenylthiouracil (PhTU). Thyreostats are not neutral to the health of animals because cardiovascular disorders such as accelerated heart rate, dyspnoea, and apnea may occur after their administration. There is also hypertrophy of the thyroid gland in animals receiving thyreostatic drugs. It was found that the average weight of the thyroid gland in calves is about 16 g, while in young and adult cattle, it is 25 g. According to some researchers, gland not exceeding 50 g falls within the normal range, while others accept an upper limit of 60 g (24). A higher thyroid weight may indicate the use of thyreostatic substances in cattle and is one of the preliminary parameters obtained in investigation of any illegal administration of these compounds to animals. Thyroid hypertrophy in animals may also be a consequence

Table 1. Structures of investigated compounds

Tapazole (TAP)		Formula M W CAS	$C_4H_6N_2S$ 114.17 60-56-0
Thiouracil (TU)		Formula M W CAS	$C_4H_4N_2OS$ 128.15 141-90-2
Methylthiouracil (MTU)		Formula M W CAS	$C_5H_6N_2OS$ 142.18 56-04-2
Propylthiouracil (PTU)		Formula M W CAS	$C_7H_{10}N_2OS$ 170.20 51-52-5
Phenylthiouracil (PhTU)		Formula M W CAS	$C_{10}H_8N_2OS$ 204.25 36822-11-4
Dimethylthiouracil (DMTU)- IS		Formula M W CAS	$C_6H_8N_2OS$ 156.20 28456-54-4

of environmental factors (iodine deficiency) as well as a diet rich in cruciferous plants (*Brassicaceae, Cruciferae)* (13). Another preliminary indirect method of testing the use of thyreostatic agents in the breeding of slaughter animals is a histological test, based on the assessment of hypertrophic changes in the thyroid gland (14). Due to the high percentage of false positives, the usefulness of this test is questionable. In recent years, physiochemical methods, in particular liquid chromatography with mass spectrometry (12, 26) have been of the greatest importance in the study of thyreostatic residue, making it possible to determine this group of compounds at the currently required level of 10 μg kg^{-1} (L^{-1}) (3). The necessity to maintain surveillance over the residues of thyreostatic substances results from both concern for the welfare of the consumer and legal aspects. Monitoring for illicit use of these substances is mandated by the EU Directive 96/23/EC (7), which is implemented in each member state according to the national residue control plan.

In Poland, since 1990 research has been conducted to detect residues of tapazole, thiouracil, methylthiouracil, propylthiouracil, and phenylthiouracil residues (Table 1), which according to the guidelines of Directive 96/23 are classified in Group A2 as prohibited substances (7).

The Polish national residue control plan is fully compliant with European regulations, and its study principles are consistent with the Regulation of the Polish Minister of Agriculture and Rural Development of 21 June 2017 (16). These documents also define the scope of the research, the sampling strategy carried out under the programme, and the proceedings in the case of the presence of unauthorised substances. The number of animals which must be examined for thyreostat residues each year depends strictly on the number of animals slaughtered in the country in the previous year. A certain percentage of the samples must be taken at farm level in prohibited substance surveillance, and for this reason in addition to biological material, drinking water or feed samples should be collected. In Poland, more than 7,000 biological samples of animal origin are tested each year for anabolic compounds of groups A1–A4, of which about 500 are investigated for the presence of thyreostats. The paper presents the results of research conducted in Poland in 2011–2017 as part of the national residue control plan for thyreostatic drug residues in samples of animal origin.

Material and Methods

Sampling. The material for testing was urine, muscle, and drinking water samples officially collected by Veterinary Sanitary Inspectors in slaughterhouses and farms throughout the country in accordance with the

National Programme for Control of Presence of Prohibited Substances and Chemical and Biological Residues in Animals and Food of Animal Origin. The requirements of national and EU legislation specify the levels of sampling. For cattle, one half of the samples should be taken from live animals at the place of their rearing and one half at the slaughterhouse. In the case of swine, the minimum number of farms to be visited annually must represent at last one farm per 100,000 pigs slaughtered the previous year. In 2011–2017, a total of 3,491 urine samples were collected for testing for thyreostatic drugs, including 1,816 samples of bovine urine, 1,647 samples of porcine urine, 7 urine samples taken from sheep and 21 from horses. In addition, 349 muscle samples were taken from rabbits, poultry, and farmed game animals. Samples of drinking water totalling 127 were also collected on pig and poultry farms. After delivery to the laboratory, the urine samples were directly analysed or acidified with acetic acid to pH = 2 to prevent instability of the thyreostats, stored below −18°C, and then thawed before analysis. Tissue and water samples were also stored in the freezer (<−18°C) until analysis was performed.

Methods. Studies of thyreostatic drugs have been carried out since 2011 in six Polish regional laboratories (those of the Veterinary-Sanitary Inspectorates (ZHW) in Białystok, Gdańsk, Katowice, Poznań, Warsaw, and Wrocław) and in the Department of Pharmacology and Toxicology of the National Veterinary Research Institute (NVRI) in Pulawy, which acts as the National Reference Laboratory (NRL) in this field. Non-compliant results obtained by regional laboratories are confirmed in the NRL in Pulawy. Five laboratories perform tests using the same method of liquid chromatography with tandem mass spectrometry, which was developed at the NVRI (26). In brief, thyreostats were extracted from urine samples with diethyl ether after derivatisation with 3-iodobenzylbromide in basic medium (pH 8.0) and analysed by gradient elution on a Poroshell C18 column (Agilent Technologies, USA) with triple quadrupole MS detection with turbo spray source. Thyreostats were extracted from muscle tissue with methanol/Britton-Robinson buffer (pH 8.0), afterwards the denaturation of matrix protein was performed, and then the same steps as for the urine, samples were carried out. The method was validated in accordance with the Commission Decision 2002/657/EC (1), as required in residue studies in products of animal origin. The procedure used offers low detection limits, well below the currently proposed recommended concentration of thyreostatic drugs in urine and in tissues, which is 10 μg L^{-1} (kg^{-1}) (3). The method was verified with positive results in a proficiency study organised in 2012 by the Food Analysis Performance Assessment Schemes (FAPAS) and in 2013 by the European Reference Laboratory at the Institute of Food Safety (RIKILT) in Wageningen in which the NRL was involved. One laboratory which participated in the research also used the LC-MS/MS method, but with a different sample preparation protocol (17, 18). In the interlaboratory studies organised three times by the National Reference Laboratory in Pulawy, all participants obtained satisfactory results, so the applied methods are reliable and guarantee accurate results.

The performance of the method used for confirmatory purposes for thyreostats detection in urine, water, and muscle samples is presented in Table 2.

Results

In 2011–2017, a total of 3,967 samples were examined in Poland for thyreostatic drug residues. The results of the examinations are presented in Table 3.

Table 2. Method trueness and precision for thyreostatic drugs in urine, muscle (26), and drinking water samples

Compound	Linear range (μg L^{-1}) (μg kg^{-1})	Apparent recovery (%)	Repeat-ability (R.S.D.,%)	Reproduci-bility (R.S.D.,%)	Decision limit CCα (μg L^{-1}) (μg kg^{-1})	Detection capability CCβ (μg L^{-1}) (μg kg^{-1})	Uncertainty U (%)
Tapazole							
urine	0.98 – 50	96	9.3	13.8	0.98	1.67	46
muscles	1.29 – 50	85	15.2	29.7	1.29	2.20	46
water	1.81 – 50	94	9.6	23.6	1.81	3.08	30
Thiouracil							
urine	0.91 – 50	97	7.3	10.2	0.91	1.56	26
muscles	0.73 – 50	87	2.1	3.9	0.73	1.25	30
water	1.31 – 50	104	4.9	11.4	1.31	2.23	29
Methylthiouracil							
urine	0.84 – 50	107	7.4	7.9	0.84	1.43	45
muscles	0.70 – 50	94	2.5	5.1	0.70	1.20	30
water	1.11 – 50	108	5,9	5.9	1.11	1.89	19
Propylthiouracil							
urine	1.24 – 50	117	8.0	8.0	1.24	2.11	29
muscles	0.57 – 50	84	2.1	4.0	0.57	0.97	22
water	1.42 – 50	105	5.2	5.7	1.42	2.42	25
Phenylthiouracil							
urine	0.89 – 50	109	8.7	15.0	0.89	1.52	44
muscles	0.83 – 50	95	2.7	2.9	0.83	1.42	21
water	1.78 – 50	91	12.2	18.3	1.78	3.03	22

Table 3. The results of testing for thyreostatic drugs in biological material of animal origin in Poland in 2011–2017

Species	Sampling place	Matrix	Number of samples tested	Number of non-compliant samples/results	Concentrations of compounds detected (μg L^{-1})/year
Cattle	farm	urine	947	1/1	22.0 ± 5.7 (TU)/2015
	slaughterhouse	urine	863	3/3	15.5 ± 4.0 (TU)/2012 40.4 ± 10 (TU)/2013 22.0 ± 5.7 (TU)/2014
Suspect samples	farm	urine	5	0	
	slaughterhouse	urine	1	0	
Swine	farm	water/urine	78/59	0	-
	Slaughterhouse	urine	1,585	4/5*	16.6 ± 4.4 (TU)/2011 15.9 ± 4.0 (TU)/2013 23.7 ± 5.9 (TU)/2013 5.1 ± 2.3 (MTU)/2015 3.4 ± 0.9 (PTU)/2015
Suspect samples	farm	urine	1	0	
	slaughterhouse	urine	2	0	
Sheep/goats	slaughterhouse	urine	7	0	
Horses	slaughterhouse	urine	21	0	
Rabbits	slaughterhouse	muscle	2	0	
Chicken	farm	water/muscle	31/20	0	
	slaughterhouse	muscle	284	0	
Other poultry	farm	water	18	0	
	slaughterhouse	muscle	36	0	
Farmed game	slaughterhouse	muscle	7	0	
Summary			3967	8/9 (0.23%)	

* – one sample was non-compliant for two substances, therefore the number of non-compliant results is higher than the number of non-compliant samples of the same group

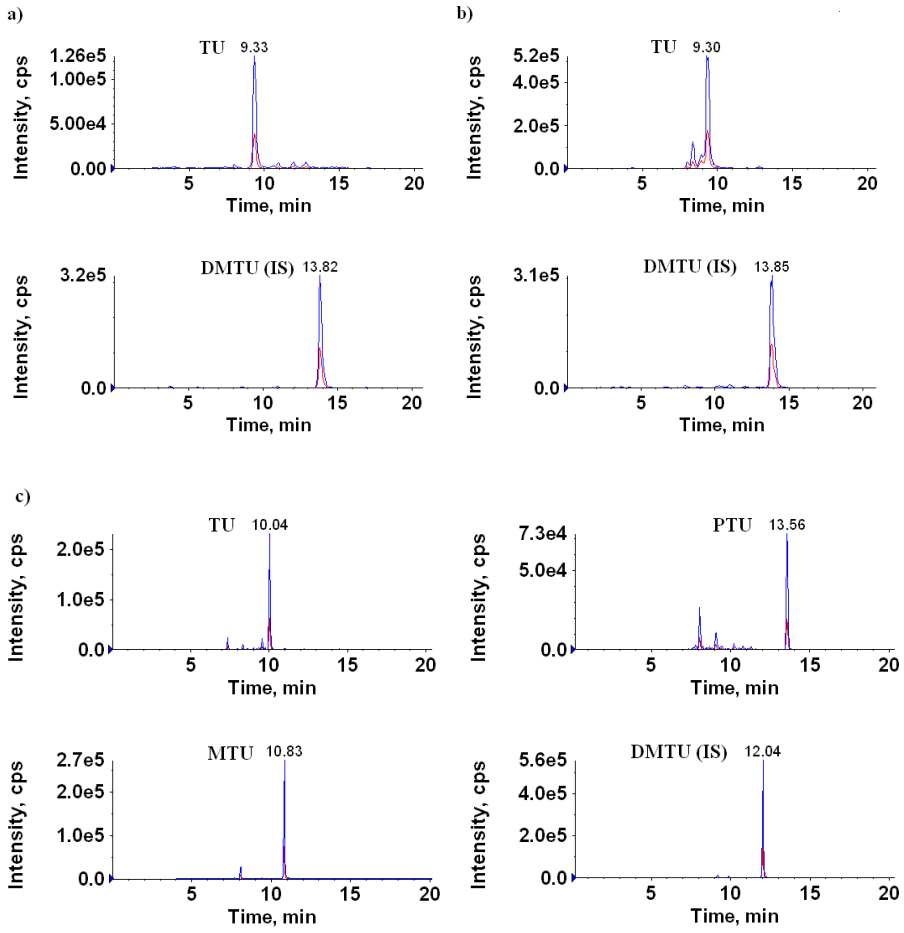


Fig. 1. LC-MS/MS MRM chromatograms of a) a bovine urine sample spiked at 10 μg L^{-1} with TU; b) a real urine bovine sample confirmed at 40.4 ± 10.0 μg L^{-1} of thiouracil; c) a real urine sample from a pig confirmed at 6.5 μg L^{-1} of thiouracil, 5.1 ± 2.3 μg L^{-1} of methylthiouracil, and 3.4 ± 0.9 μg L^{-1} of propylthiouracil

Of the 947 urine samples collected from cattle on farms, thiouracil exceeded the recommended concentration (RC) of 10 μg L^{-1} in only one. Three cattle urine samples out of 863 taken at the slaughterhouse contained thiouracil with concentrations of 15 to 40 μg L^{-1}. In three urine samples taken from pigs at the slaughterhouse, thiouracil was found and its concentration exceeded 10 μg L^{-1}. In one sample of porcine urine, in addition to TU with a concentration below the RC (6.5 μg L^{-1}), methylthiouracil and propylthiouracil were also detected above the decision limits of the method.

A total of 3,491 urine samples were tested, of which eight were non-compliant with the applicable regulations, representing 0.23%. Fig. 1 shows chromatograms of urine samples in which thyreostatic compounds were found. The thyreostatic substances were not detected in samples of drinking water taken on poultry or pig farms or in muscle samples from chickens, turkeys, ducks, rabbits, or farmed game animals. Also in the samples taken again during the investigation (suspect sample), no thyreostatic agents were found.

Discussion

The effective control of chemical residues in food is not only to protect the health of consumers but also to meet the requirements of international food trade. In the EU Member States, there are uniform rules for organising and conducting the monitoring of residues of substances showing anabolic effect, veterinary drugs, and environmental contaminants in animal tissues, food of animal origin, and in animal nutrition, which are included in the Council Directive 96/23/EC (7) and new other legal acts (15). The assumptions of the residue testing programme and its plan are prepared annually at the National Veterinary Research Institute, approved for implementation by the Chief Veterinary Officer, and then evaluated and accepted by the European Commission. Every year, around 30,000 samples are analysed under this programme, including 500–600 samples for thyreostatic agent determination. In Poland, regular tests of thyreostats in animal tissues have been conducted since 1990. According to Annex II of the Directive 96/23/EU, thyreostatic drugs (Group A2) should be tested in cattle, pigs, sheep, goats, horses, rabbits, farmed game animals, and poultry. However, taking into account the legitimacy of administration of thyreostatic drugs to poultry, a gentleman's agreement was entered into at the meeting of the Residue Expert Working Group in May 2012 in Brussels at the European Commission not to require testing of poultry for thyreostats (4). Therefore, since 2013, no thyreostatic drugs have been tested for in poultry in Poland. Prior to 2013, no positive result for this species was reported. The introduction of a very sensitive method for routine detection of thyreostats (12, 23) generated a 50-fold reduction in detection limits and caused some European countries including Poland to detect thiouracil in urine samples of slaughter animals (11, 25, 27). The research undertaken in Poland from June 2010 to July 2011 shows that 14.3% of urine samples contained thiouracil above the 0.91 μg L^{-1} decision limit of the applied method, including 1.49% of samples over the recommended concentration of 10 μg L^{-1} (27). Research conducted in the last seven years shows that the number of non-compliant results has decreased significantly, and only 0.23% of samples fail. The reason for this drop was the introduction from 2016 of a higher value of decision limit for control purposes of 30 μg L^{-1} for TU, which cut non-compliant results in the last two years. The problem with thiouracil is that there are currently no methods to distinguish whether the compound was illegally administered to animals or has an endogenous nature. Determination of whether the compound is exogenous or of natural origin can be made based on knowledge of marker metabolites. Therefore, in some scientific centres, the search for markers to distinguish between endogenous and exogenous nature is currently being undertaken, and the recent work of Belgian scientists in this direction is very promising (21). Another solution to this problem is the determination of the threshold value in a population study, above which it is considered statistically unlikely that the result could be produced "naturally" without producing a high rate of false compliance (19).

Estimated on the basis of epidemiological studies conducted in six European countries including Poland, the threshold value for natural thiouracil concentration in cattle urine can be as high as 18 μg L^{-1} (99^{th} percentile) (25). Therefore, the EU Reference Laboratory at RIKILT (Wageningen, the Netherlands) which is responsible for compounds with anabolic activity proposed a new recommended concentration of 30 μg L^{-1} for thiouracil in place of the current 10 μg L^{-1} (20), and this level of action for this compound was introduced in the national residue control plan. New criteria are expected to be officially established for the natural levels of this compound in the urine of slaughter animals. The investigation conducted by the Veterinary Inspectorate did not show that thiouracil was illegally administered to animals. During the explanatory proceedings, additional samples were taken from the place of origin of the animals in which thiouracil was detected, and no presence of this compound was found in these suspect samples. Therefore, it was considered that the presence of thiouracil resulted most probably from feeding diets rich in cruciferous plants. According to the literature, thiouracil may occur naturally in some animal species, *inter alia,* following the inclusion of cruciferous plants in their diet (13), which includes rape widely fed to farm animals in Poland. The use of rapeseed meal in excess of the established standards (30%) in compound feed may be the cause of elevated values of urinary thiouracil concentrations. The mechanism of formation of this compound has not been fully elucidated so far, and not all factors favouring the formation of thiouracil in animals and humans are

known. In addition to thiouracil, two other compounds of the thyreostatic group, methylthiouracil and propylthiouracil were found in one urine sample collected from a pig. The investigation carried out by the Veterinary Inspectorate did not give any grounds for stating that these compounds were given to animals. The presence of thiouracil analogues most probably resulted from feeding diets rich in cruciferous plants, which is all the more likely given that thiouracil was also found in this sample but below the RC of 10 μg L^{-1}. Until now, there has been no news on the endogenous nature of these compounds in the world literature. Additionally, according to the EFSA report, one more derivative of thiouracil, 5-methyl-2-thiouracil has been detected in the urine of cattle in Europe (8). For several years, thiouracil has been at the centre of attention of scientists involved in the study of anabolic compound residues in animal tissues. The reason for this focus is that it is the most frequently detected compound with anabolic properties banned for use in slaughter animals. The percentage of non-compliant samples reported in relation to the total number of targeted samples analysed for the thyreostats group in Europe over the period 2011–2015 ranged from 0.33% to 0.77% with the highest value reported in 2013. It should be added that in this period the average number of non-compliant results for the whole group A is only 0.10% (8). Similarly in Poland, it was in 2013 when the highest percentage of non-compliant results was recorded (0.66%). Recently, however, decreases in the number of samples non-compliant for antithyroid agents have been noted compared to previous years, and in 2016, according to European data only 0.45% of samples tested did not meet the required criteria (9). This is probably due to the introduction of a new threshold value for thiouracil proposed by the EURL RIKILT in Wageningen and its implementation in the control plan in some European countries.

Collating the presented results, it should be noted that the percentage of non-compliant results is slightly lower in Poland than the European average. The system of monitoring the residues of compounds showing an anabolic effect in slaughter animals in Poland is being constantly improved, and the applied research methods meet the high requirements imposed by European regulations guaranteeing high quality of conducted research. Because the number of results inconsistent with the applicable regulations is negligible, it can be concluded that Polish breeders adhere to the ban on the use of thyreostatic drugs in the fattening of slaughter animals. Regular tests carried out for many years make it possible to assess that Polish food of animal origin is safe for the consumer in relation to this group of compounds.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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