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Determination of thyreostat residues from bovine matrices using high-performance liquid chromatography

Robert K. Buick*, Caroline Barry, Imelda M. Traynor, W. John McCaughey, Christopher T. Elliott

Department of Agriculture for Northern Ireland, Veterinary Science Division, Stoney Road, Belfast BT4 3SD, Northern Ireland, UK

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Abstract

High-performance liquid chromatography (HPLC) methodologies were evaluated for the detection and quantification of thyreostatic drug residues in cattle serum and thyroid tissue. The paper details a protocol, using a simple ethyl acetate extraction for the determination of thiouracil, tapazole, methyl thiouracil, propyl thiouracil and phenyl thiouracil in thyroid tissue. Using two sequential HPLC injections, and quantitative analysis, in two steps, all five thyreostats were detectable at concentrations greater than 2.45–4.52 ng/g. Modifications to a published method for detection of thyreostatic residues in serum involving the addition of mercaptoethanol and a freezing step are described. The modifications improved sensitivity and allowed detection of the five thyreostats at levels greater than 16.98–35.25 ng/ml. Young bulls were treated with thyreostats to demonstrate the validity of the methodologies described. Administered thyreostats were not absorbed equally by the test animals and the compounds were not all detected in the serum samples removed at 7 days following drug withdrawal. These experiments indicate the necessity to be able to detect thyreostat residues in a variety of matrices.

Keywords: Thyreostat

1. Introduction

Thyreostats or thyroid-inhibiting compounds are orally active drugs which may be used to increase the weight of cattle prior to slaughter. This weight gain is due mainly to increased water absorption from within the gastro-intestinal tract and water retention within tissue [1,2]. Although propylthiouracil has been used in the treatment of hyperthyroidism, the uncontrolled introduction of this and other thyreostats into the human food chain could have serious health implications. Consequently specific legislation has been promulgated within the European Union prohibiting the use of thyreostats in animal production [3,4].

Various techniques have been described in the literature for the detection or determination of thyreostat abuse in animal matrices. These range from weight measurement of the thyroid gland [5], to high-performance thin layer chromatography (HPTLC) [6–8] and high-performance liquid chromatography (HPLC) with various forms of detection [9–11]. The reference screening method for thyreostat detection, as published by the European Union

*Corresponding author.

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[12], is based on the methodologies of De Brabander and co-workers [13,14]. This complicated method relies on the extraction of thyreostatic compounds from a variety of tissue matrices, thyroid, kidney and liver, clean-up through a mercurated ion-exchange column, derivatization of the compounds, followed by separation and identification by two-dimensional HPTLC.

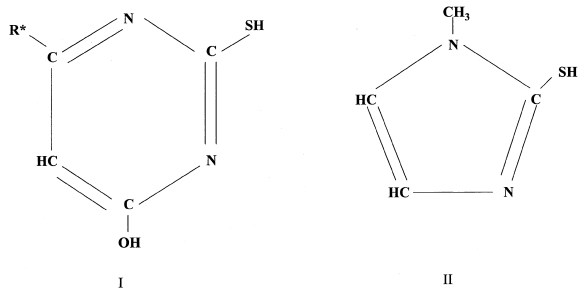
Recent regulations within the European Union [15] will result in increased monitoring for thyreostats and other illegal substances. To deal with this large increase in sample numbers, simplified screening methods, for thyreostat determination which do not require complicated separation or derivatization stages would be advantageous. Existing HPLC methodologies [10] are suitable for accurate screening of bovine serum for thyreostats. However, since many samples are taken at slaughter the availability of a quick thyroid screening method is necessary.

This paper describes an HPLC method for determination of thyreostatic residues in thyroid tissue, and also outlines modifications to the method of Moretti et al. [10] for determination of their residues in serum. To assess the applicability of these methods, to incurred samples, various combinations of thyreostats were administered in feed to young cattle over a period of time and samples of serum and tissues were taken during and subsequent to the administration phase for analysis.

2. Experimental

2.1. Reagents and chemicals

All solvents used were of HPLC quality and chemicals were analytical grade products and were obtained from various manufacturers. 2-Thiouracil (TU), 4(6)-methyl-2-thiouracil (MTU), 4(6)-*n*-propyl-2-thiouracil (PTU) and 4(6)-phenyl-2-thiouracil (PhTU) were obtained from Sigma (Poole, UK), 1-methyl-2-mercaptoimidazole (tapazole, TAP) was obtained from Aldrich Chemicals (Gillingham, UK). The structural formulae of thiouracil and tapazole are shown in Fig. 1. Stock solutions were prepared by dissolving the thyreostats in methanol at a concentration of 1 mg/ml, other standards were prepared as required by further diluting from these stocks, also in methanol.



R* this group may be substituted by methyl, propyl or phenyl groupings.

Fig. 1. Structural formulae of 2-thiouracil (I) and 1-methyl-2-mercaptoimidazole (tapazole) (II).

2.2. Apparatus

The HPLC apparatus comprised of a Merck–Hitachi (BDH/Merck, Lutterworth, UK), L-6500 intelligent pump system coupled to a L-4500 diode array detector (DAD). Samples were introduced via a Rheodyne valve assembly with a 50-μl sample loop. Compound separation was achieved using a LiChrosorb RP-18 chromatography column (250×4 mm I.D., 10 μl particle size) (BDH/Merck, Lutterworth, UK), preceded by a LiChroprep RP-18 guard column (BDH/Merck). Chromatographic data were recorded and analysed using a DAD system manager software package, supplied with the DAD, installed on a personnel computer.

2.3. Preparation and extraction of spiked thyroid tissue.

Thyroid glands from cattle raised from birth at an experimental farm (Agricultural Research Institute of Northern Ireland, Hillsborough, N. Ireland, UK) and known not to have been treated with thyreostats were trimmed, chopped and weighed into 5-g amounts. All five thyreostats were added from the methanolic standard solutions to each 5-g sample to give the equal thyreostat concentrations in each tissue sample. Samples were prepared to give individual thyreostat concentrations of 0, 0.075, 0.15, 0.5, 1.0 and 1.5 μg/g .

To each 5-g tissue sample was added 200 μl 0.1

M EDTA (disodium salt). Samples were homogenised with 15 ml of ethyl acetate for 1 min at full speed in a homogeniser (Ultra-Turrex, BDH/Merck), followed by a thorough mixing for 15 min using an end-over-end shaker (Luckham Ltd., Burgess Hill, UK). The samples were then centrifuged at 3500 *g* for 15 min at 10°C and placed in a liquid nitrogen cooling block to freeze the aqueous layer allowing clean separation of the organic layer which was decanted and evaporated to dryness at 60°C under a stream of nitrogen. The resultant residue was redissolved in 2.5 ml of chloroform for clean up.

To clean up the tissue residues, silica gel columns (1 g, 6 ml), (Baker, Milton Keynes, UK) were conditioned with 2×5 ml of chloroform. The chloroform extract was added to the column and the sample tube rinsed with a further 2.5 ml of chloroform, which was also added to the column. The column was further washed with 5 ml of chloroform and eluted with 6 ml of 15% methanol–85% chloroform. The elutant was evaporated to dryness at 60°C under nitrogen and the residue redissolved in 200 μl of methanol for HPLC analysis.

2.4. Preparation and extraction of spiked serum

Serum samples from cattle raised at an experimental farm (Agricultural Research Institute of Northern Ireland), and known not to have been treated with thyreostats were divided into 0.5-ml aliquots. All five thyreostats were added from the methanolic standard solutions to each 0.5-ml aliquot to give equal thyreostat concentrations in each aliquot. Samples were prepared to give individual thyreostat concentrations of 0, 0.15, 0.25, 0.5, 1.0 and 1.5 μg/ml.

Serum samples were treated according to the method of Moretti et al. [10] with the following modifications. To aid recovery of the added thyreostats, 15 μl of mercaptoethanol were added to the serum samples (J. Blanchflower, D.A.N.I., Veterinary Science Division, Belfast, pers. commun.). Additionally, after homogenisation and centrifugation, the aqueous serum layer was quickly frozen in a liquid nitrogen cooling block to ensure full and clean separation of the organic phase.

Extracted serum samples did not require further clean-up and were evaporated to dryness and resuspended in 200 μl of methanol for HPLC analysis.

2.5. Preparation and extraction of incurred positive material.

Eight intact male Friesian calves, approximately 6 months old, were housed in pairs (groups A, B, C, D). For 55 consecutive days each group of calves received a mixture of two different thyreostats once daily. A total of 10 g of thyreostat was added to 2 kg of concentrated calf feed. The calves in each group were fed together and were not allowed normal forage until all concentrate had been consumed. Each group received a different combination of thyreostatic mixtures. The calves in group A received 5 g of TU and 5 g TAP daily, group B received 5 g TAP and 5 g MTU daily, group C received 5 g MTU and 5 g PTU daily, while group D received 5 g PTU and 5 g TU daily. PhTU was not used in this incurred experiment principally due to the high cost of the material. During the medication period regular blood samples were taken from all calves (Table 3). Samples were allowed to clot, centrifuged for 10 min at 1500 *g*, and the serum separated and frozen at −20°C until analysis. Following drug withdrawal serum levels were monitored until all thyreostats were no longer detectable in the samples. At this point one calf from each pair was slaughtered and the thyroid glands removed. The remaining calves were retained for a further 3-week period, at which point they were also slaughtered and the thyroid glands removed. All thyroids were stored at −20°C prior to analysis.

3. Chromatographic conditions

3.1. Tissue

To separate and quantify TU, MTU, PTU and PhTU a non-linear gradient using acetonitrile and 0.025 *M* phosphate buffer, pH 3.0, was employed. The gradient ranged from 0% acetonitrile at $T=0$ min to 1% acetonitrile at $T=9$ min and 44% acetonitrile at $T=35$ min. A second injection was employed for the separation and quantification of TAP with a gradient of 5% acetonitrile at $T=0$ min, to 35% acetonitrile at $T=20$ min. Analysis wavelength was 276 nm for TU, MTU, PTU and PhTU, and 258 nm

for TAP. In both cases the injection volume analysed was 10 μl and the flow rate was 1 ml/min.

3.2. Serum

The chromatographic conditions described by Moretti et al. [10] were used for the analysis of thyreostatic residues in serum.

4. Results and discussion

4.1. Thyreostat determination in spiked tissue

Fig. 2a and b show typical chromatographic traces of extracted negative and spiked thyroid samples containing TU, MTU, PTU and PhTU. The presence of two major natural eluted peaks (labelled a and b) can be clearly seen early in the negative chromatogram at approximately 8 and 11 min. Under the gradient conditions chosen these compounds would co-elute with TAP and altering gradient conditions only caused co-elution with either TU or MTU. Hence it was necessary to use a second sample injection using alternative gradient conditions to successfully isolate and quantify TAP. Fig. 2c and d show the same negative and spiked thyroid matrices analysed for TAP at 258 nm using a second injection at the alternative gradient.

Retention times of the thyreostats were consistent throughout all analyses and were sufficiently separated to allow definitive identification. The mean retention times were TU 7.01 min (S.D. 0.46, $n=60$), TAP 5.75 min (S.D. 0.42, $n=60$), MTU 14.13 min (S.D. 1.1, $n=60$), PTU 23.79 min (S.D. 1.01, $n=60$) and PhTU 28.22 min (S.D. 1.2, $n=60$).

The method was validated by extraction of four replicates at each thyreostat concentration on three different occasions. The coefficient of variation within extractions (intra-assay variation) and between extractions (inter-assay variation) are shown in Table 1. The relationships between absorbance and concentration in the extracted samples over the concentration range chosen were linear with regres-

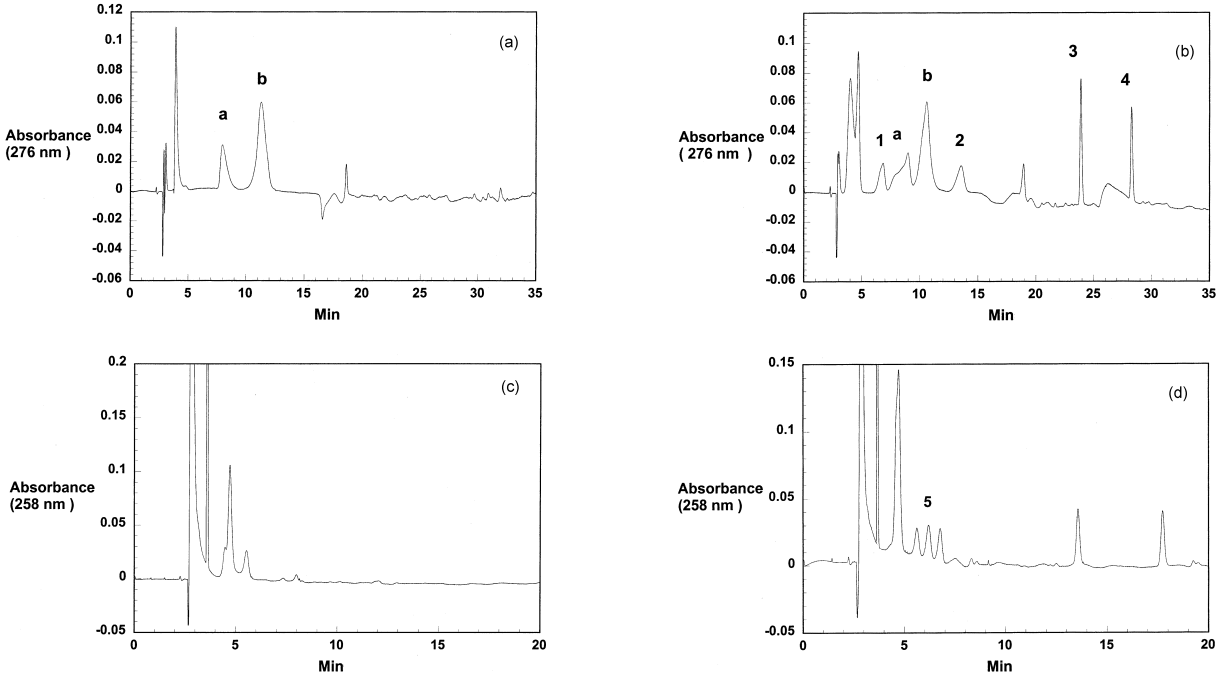


Fig. 2. Chromatograms from negative (a) and spiked (b) thyroid tissue showing natural eluted compounds a and b with eluted thyreostats, 1-TU, 2-MTU, 3-PTU and 4-PhTU. Also shown are the same samples (c) and (d) analysed using the alternative gradient conditions, as described in the text, with the clear elution of 5-TAP.

Table 1
Correlation between absorbance and concentration using the thyroid extraction method and average recoveries of thyreostats using the methodology

Thyreostat	Regression coefficient between absorbance and concentrations (r^2)	Limits of detection (LOD) and Quantification (LOQ)[a] (μg/g)		Average % recovery of thyreostat at each level studied		Coefficients of variation (%)	
		LOD	LOQ	μg/g	%	Intra-assay (n=4)	Inter-assay (n=3)
Thiouracil	0.99	0.00425	0.00480	0.075	58.66	18.67	5.68
				0.15	50.00	17.90	10.41
				0.5	46.00	11.12	11.10
				1.0	41.90	14.88	4.38
				1.5	45.84	13.86	1.94
Tapazole	0.98	0.00329	0.00372	0.075	32.00	19.37	5.68
				0.15	18.00	7.86	10.41
				0.5	26.40	3.27	7.23
				1.0	28.50	14.72	7.45
				1.5	31.20	14.24	18.27
Methylthiouracil	0.98	0.00452	0.00511	0.075	58.67	18.25	8.04
				0.15	48.67	4.94	17.96
				0.5	54.00	7.01	25.22
				1.0	54.60	13.20	25.88
				1.5	41.22	5.19	24.97
Propylthiouracil	0.99	0.00262	0.00296	0.075	52.00	19.71	18.39
				0.15	67.33	3.07	17.39
				0.5	75.40	4.02	13.45
				1.0	72.30	4.70	16.16
				1.5	68.73	2.77	11.49
Phenylthiouracil	0.98	0.00245	0.00277	0.075	36.00	19.60	7.81
				0.15	42.00	6.81	14.29
				0.5	48.00	9.52	18.14
				1.0	48.50	15.37	21.05
				1.5	48.47	14.43	18.53

[a] The limits of detection and quantification were calculated by the method suggested in European Commission Decision 93/256/EEC, i.e., the apparent analyte concentration in 20 blank samples plus three times the standard deviation of the mean (LOD) and plus six times the standard deviation (LOQ).

sion coefficients (r^2) of 0.98–0.99 (Table 1). Analytical recoveries ranged from 26.40 to 75.40% (Table 1) and are superior to the post clean up and derivatization results of De Brabander and Verbeke [6]. The limits of detection (LOD) show that the method is extremely sensitive ranging from 2.45 to 4.52 ng/g. The levels of detection of this method are comparable with the 10 ppb or better in tissue quoted by De Brabander and Verbeke [6]. However, compared to that technique, this present HPLC method uses a much simpler extraction stage and does not require derivatisation for detection.

4.2. Thyreostat determination in spiked serum

Fig. 3a–c are typical chromatographic traces from extracted negative and spiked serum samples containing TU, TAP, MTU, PTU and PhTU. Compared with extracted thyroid samples, interfering natural compounds are not a feature of the serum traces. This absence of co-elution allowed all thyreostats to be detected by one injection with a single gradient.

Retention times of the thyreostats were again very consistent and reproducible throughout all the analyses. Retention times were TU 4.33 min (S.D. 0.16,

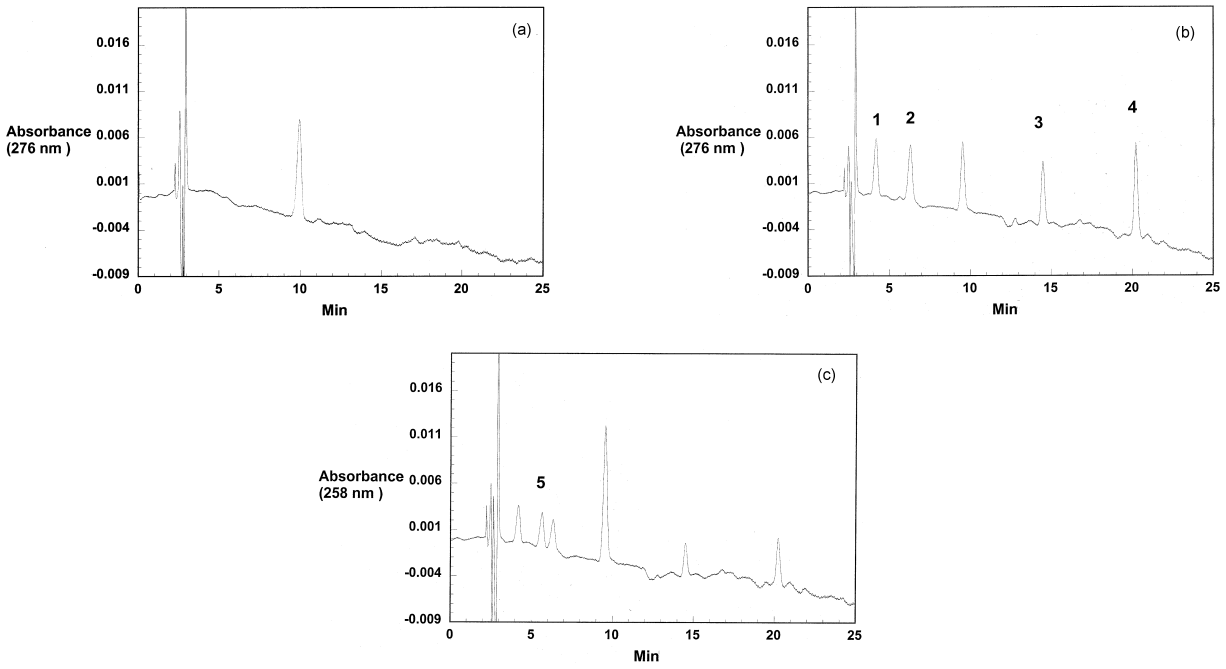


Fig. 3. Chromatograms from negative (a) and spiked (b) serum samples showing eluted thyreostats 1-TU, 2-MTU, 3-PTU and 4-PhTU. Also shown is the same spiked chromatogram reanalysed at 258 nm with 5-TAP apparent (c).

$n=60$), TAP 5.92 min (S.D. 0.26, $n=60$), MTU 6.62 min (S.D. 0.29, $n=60$), PTU 14.92 min (S.D. 0.34, $n=60$) and PhTU 20.69 min (S.D. 0.38, $n=60$).

The addition of mercaptoethanol, which as a reducing agent decreases covalent binding of the thyreostats to proteins, and using a freezing step resulted in increased recoveries for TU, MTU, PTU and PhTU, (55.20–92.60%, Table 2), compared to those previously quoted [10]. Only TAP failed to give improved recovery with these modifications. The LOD of the method ranged from 16.98–35.25 ng/ml (Table 2). While these limits were much higher than those observed for thyroid tissue they were lower than the 200 μg/l quoted by Moretti et al. [10]. This method was also validated as described above and the intra- and inter-assay variations are also shown in Table 2.

4.3. Incurred thyreostats: serum

The mean levels of thyreostats in the serum samples from the four treatment groups (A–D) of paired experimental animals are shown in Fig. 4. The results indicated that not all thyreostats were absorbed equally into the bloodstream despite being present in equal quantities in the feed material. Four thyreostats were used in this part of the study (TU, TAP, MTU and PTU).

Of these four, TAP, MTU and PTU were strongly absorbed reaching concentrations as high as 32 μg/ml in the serum of individual animals. In contrast TU only reached concentrations of <4 μg/ml within the first week of administration then stabilising at concentrations of <1 μg/ml. The combinations of thyreostats administered did not affect absorption, i.e., TU administered with TAP was equally poorly absorbed when compared to TU administered with PTU. Some of this differential absorption could have been due in part to effects from the co-administration of thyreostats or the uneven feeding patterns of the pairs of animals.

Detectable concentrations of thyreostats were found after 1 day of the onset of administration and rose to their maximum concentrations within 6–7 days. Following withdrawal of treatment, concentrations declined rapidly and were not detectable

Table 2
Correlation between absorbance and concentration using serum extraction method and average recoveries of thyreostats using the methodology

Thyreostat	Regression coefficient between absorbance and concentration (r^2)	Limits of detection (LOD) and quantification (LOQ)[a] (μg/ml)		Average % recovery of thyreostat at each level studied		Coefficients of variation (%) (n=4 intra-assay, n=3 inter-assay)	
		LOD	LOQ	μg/g	%	Intra-assay	Inter-assay
Thiouracil	0.99	0.01698	0.01919	0.15	68.00	9.75	20.27
				0.25	55.20	10.02	8.69
				0.50	66.40	5.88	11.30
				1.00	69.00	8.33	20.47
				1.50	72.40	13.08	17.99
Tapazole	0.98	0.02428	0.02745	0.15	53.33	12.70	1.79
				0.25	40.00	15.93	11.54
				0.50	50.80	2.76	15.68
				1.00	45.80	12.00	18.47
				1.50	55.47	7.18	26.67
Methylthiouracil	0.99	0.02512	0.02840	0.15	66.67	8.23	10.60
				0.25	60.80	19.58	9.57
				0.50	65.60	10.91	4.61
				1.00	69.60	6.11	15.82
				1.50	68.00	4.16	18.82
Propylthiouracil	0.99	0.03525	0.03985	0.15	76.00	11.37	4.30
				0.25	79.20	4.70	5.54
				0.50	83.20	12.41	11.45
				1.00	78.60	10.22	14.86
				1.50	79.87	0.75	11.90
Phenylthiouracil	0.99	0.02868	0.03242	0.15	74.67	11.33	9.93
				0.25	72.00	18.62	8.24
				0.50	92.00	7.84	0.25
				1.00	92.60	3.54	4.18
				1.50	91.60	6.74	5.61

[a] The limits of detection and quantification were calculated by the method suggested in European Commission Decision 93/256/EEC, i.e., the apparent analyte concentration in 20 blank samples plus three times the standard deviation of the mean (LOD) and plus six times the standard deviation (LOQ).

after 7 days. This pattern would confirm that while serum is an excellent matrix for detection of thyreostat abuse 'on farm', it is not suitable for detection at slaughter if the animals had been withdrawn for more than 7 days previously.

4.4. Incurred thyreostats: thyroid tissue

The concentrations of accumulated thyreostats in the thyroid glands of the calves slaughtered at 62 and 82 days, i.e., 7 and 27 days after cessation of administration, are shown in Table 3. While it is difficult to draw firm conclusions from single animal results several trends seem to exist. In general accumulation of thyreostats in the thyroid gland tissue occurred at low levels. The maximum concentration recorded in the tissue of 5.4 μg/g was significantly lower than concentrations circulating in the bloodstream of the calf during administration. Additionally a high serum concentration may not correlate with a high thyroid concentration. TAP which reached high levels in the serum only accumulated at a concentration <1 μg/g in the thyroid. TU concentrations were low in both serum and thyroid

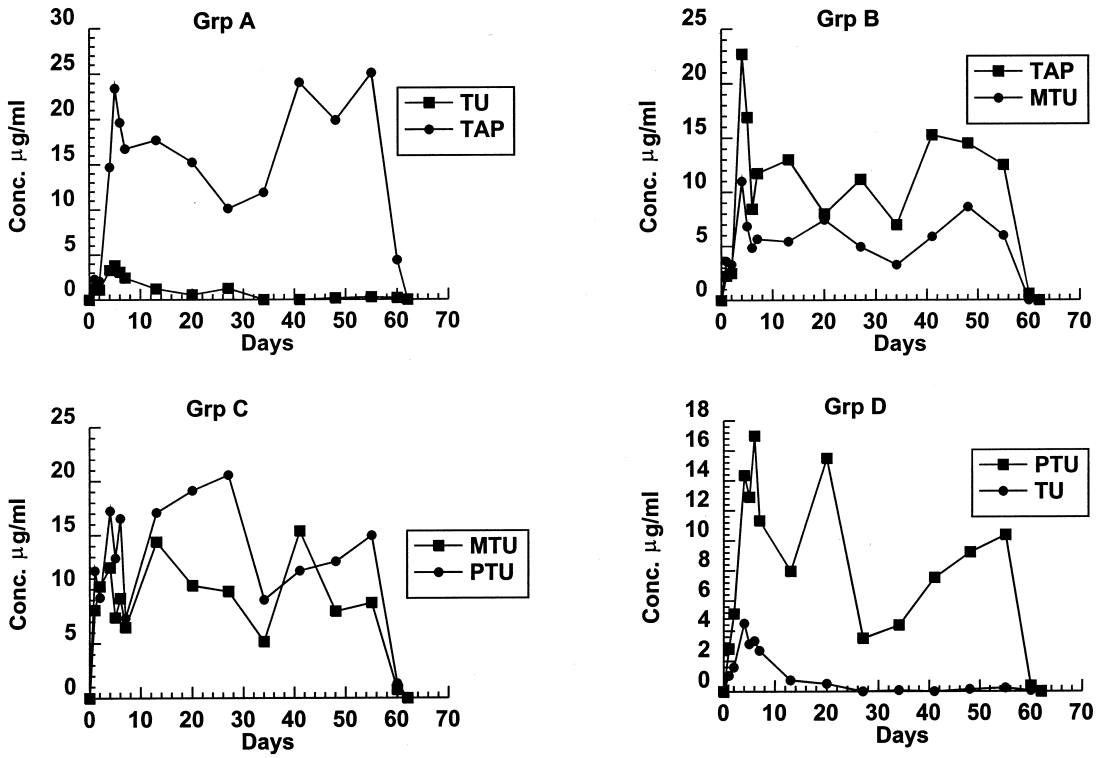


Fig. 4. Average thyreostat levels in the serum of pairs of calves administered with differing combinations of thyreostats. The graphs show the rapid but differential absorption of the compounds following onset of treatment and the rapid decrease in serum levels following treatment cessation.

indicating poor absorption of TU from the digestive tract. Concentrations of MTU and PTU were still detected in the thyroid after 27 days withdrawal, confirming that when significant levels of thyreostat were accumulated in the thyroid tissue these may be persistent. These results would indicate that, because of the differential uptake and accumulation of the thyreostats, the choice of thyreostat used in abuse

Table 3
Levels of accumulated thyreostat in thyroids of experimental animals.

Treatment group	Thyreostats administered to calf	Concentration in calf killed after 7 days withdrawal of thyreostats from feed (µg/g)	Concentration in calf killed after 27 days withdrawal of thyreostats from feed (µg/g)
A	Thiouracil	N.D.[a]	N.D.[a]
	Tapazole	0.28	N.D.[a]
B	Tapazole	N.D.[a]	N.D.[a]
	Methylthiouracil	0.456	N.D.[a]
C	Methylthiouracil	5.417	0.065
	Propylthiouracil	3.077	N.D.[a]
D	Propylthiouracil	0.086	N.D.[a]
	Thiouracil	0.078	0.074

[a] N.D., not detected.

could affect the likelihood of that abuse being detected at slaughter.

5. Conclusion

Current sampling regimes within the European Union necessitate that thyreostat screening methodologies must be able to accommodate a variety of animal matrices. The rapid disappearance of thyreostatic drugs from the serum after withdrawal from the diet, necessitates that reliable tissue detection methods are also required to detect drugs that may have been previously administered but withdrawn pre slaughter. The thyroid method described is an improvement over others previously published since HPLC provides both qualitative and quantitative analysis in one step, and additionally successfully quantifies all five major thyreostats following simple extraction and clean-up stages. The disadvantage from using two injections and gradients is offset by the reproducibility and sensitivity of the method.

This work has demonstrated that the method of Moretti et al. [10], modified as detailed, is an effective method to detect and quantify levels of TU, TAP, MTU, PTU and PhTU in serum and would be useful for on-farm testing. However, the animal experiment confirmed the necessity to have a post-slaughter tissue test to detect past abuse. We have also shown that co-administration of the major thyreostats does not result in equal absorption into the bloodstream and accumulation in thyroid tissue. This finding could have implications for the detection of abuse of these compounds.

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References

[1] G. Terplan, L. Kothler, B. Rolle, H. Geist, Fleischwirtschaft 44 (1964) 457.
[2] W. Wöhlbier, W. Schneider, Z. Tierphysiol. 21 (1966) 34.
[3] E.C. Directive 81/602, Off. J. Eur. Commun., 32 (1981) Document number L 222.
[4] E.C. Directive 86/649, Off. J. Eur. Commun., 36 (1986) Document number L 275.
[5] J.G. Vos, R.W. Stephany, J.W. Caspers, J.th.G. Van Coon, J.W.H. Metzlar, H.B.M. Overhaus, Vet. Q. 4 (1982) 1.
[6] H.F. De Brabander, R. Verbeke, J. Chromatogr. 108 (1975) 141.
[7] G. Moretti, M. Amici, P. Cammarata, F. Fracassi, J. Chromatogr. 442 (1988) 459.
[8] M.F. Pochard, M. Karageorgis, M. Chevalier, Analysis. 11 (1983) 499.
[9] H. Hooijerink, W.G. De Ruig, J. Chromatogr. 394 (1987) 403.
[10] G. Moretti, P. Betto, P. Cammerata, F. Fracassi, M. Giambenedetti, A. Borghese, J. Chromatogr. 616 (1993) 291.
[11] M.F. Pochard, M. Karageorgis, M. Chevalier, J. Chromatogr. 298 (1984) 183.
[12] R. J. Heitzman (Ed.), Veterinary Drug Residues, Residues in Food Producing Animals and Their Products, Reference Materials and Methods, Published on behalf of the Commission of the European Communities, EUR 15127-CN, 1994.
[13] H.F. De Brabander, R.Verbeke, in: Proc. 30th Eur. Meat Res. Workers, Bristol, 1984, p. 387.
[14] H.F. De Brabander, P. Batjoens, J. Van Hoof, J. Planar Chromatogr. 5 (1992) 124.
[15] E.C. Directive 96/23, Off. J. Eur. Commun. 10 (1996) Document number L 125.