8:5 Determination of methylthiouracil and analogous thyreostatic drugs

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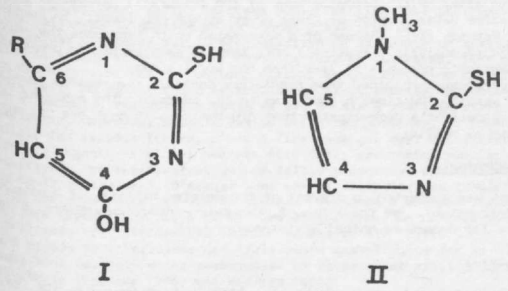
Introduction

Treatment of cattle with thyreostatic drugs is detected by the residues present in plasma, excreta, meat or organs of the animal. Optimal detection of illegal treatment with thyreostatics will be achieved through selection of the tissue or physiological fluid with the highest residue concentration and the use of a reliable sensitive detection method. A specific detection procedure of thiouracil and analogous compounds (Fig. 1), based on fluorescence induction of the NBD-derivatives (7-chloro-4-nitrobenzo-2-oxa-1,3-diazole) with cysteine,



I II

Fig. 1 : Structural formulas of thiouracil and analogous drugs
I : 4(6)-R-thiouracil (R=H (TU), Me (MTU), n-propyl (PTU) or phenyl (PhTU)
II : 1-methyl-2-mercaptoimidazole (tapazole (TAP))

has been described previously (1). This method was adopted by the BENELUX (2) and EEC (3) for qualitative analysis of these drugs at the 50 ppb level. However, the clean-up of the extracts did not permit to exploit the sensitivity of the reaction. A rapid and selective extraction procedure for thyreostatic drugs, based on a specific complex formation of the drugs with mercury ions (4), is presented here allowing a quantitative determination of the thiouracils in various extracts of biological origin.

Materials and Methods

- Preparation of the mercurated resin

Dowex 1 x 2 (50-100 mesh) is washed successively with 10 bed volumes distilled water, 0.5 N NaOH, dist. water, 0.5 N acetic acid and dist. water. The wet anion-exchanger (10 ml) is then shaken with an aqueous solution of 2,7-dibromo-4-hydroxymercurifluorescein (250 mg) during 24 hours. The mercurated resin is then washed with water until the eluate is colourless. Afterwards the resin is treated with 100 ml 0.1 N HCl in 0.5 M NaCl, washed with 500 ml dist. water, treated with 100 ml 0.1 N NaOH and finally washed with 500 ml dist. water. The mercurated resin is stored in the dark.

- Micro-column for the clean-up of thyreostatic drugs

A diagram of the chromatographic micro-column, used for the clean-up of thyreostatic drugs, is given in Fig. 2.

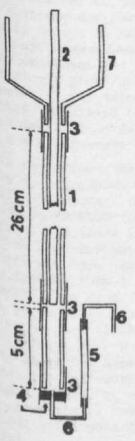


Fig. 2 : Construction of the micro-column.

1. glass column (4 mm I.D., 8 mm O.D.)
2. glass rod (3 mm O.D.)
3. silicon tubing (4 mm I.D., 6 mm O.D.)
4. silicon tubing (0.5 mm I.D., 4 mm O.D.)
5. silicon tubing (0.5 mm I.D., 1 mm O.D.)
6. teflon tubing (0.3 mm I.D., 0.7 mm O.D.)
7. glass funnel

The micro-column is prepared as follows : the column is filled with water and the glass rod is removed. Approximately 0.6 ml mercurated resin is suspended in water and added to the glass funnel. After sedimentation of the resin in the glass column to a height of 5 cm, the excess of resin is removed. After depositing the glass rod on the resin bed, the column is ready for use.

- Analytical procedure

2 g of tissue, 2 ml urine, plasma or skim milk are homogenised in 10 ml methanol using an ultra-turrax. The internal standard solution (4 (5,6)-dimethyl-2-thiouracil (DMTU), 100 µl) is added and the homogenate centrifuged at 10 000 rpm (12 000 g) during 10 minutes. The supernatant is decanted and subsequently percolated through the mercury column. The column is washed with water and the thyreostatic drugs are displaced with 5 ml elution solution (0.5 M NaCl; 0.1 N HCl; pH = 1). The eluate is neutralized (100 µl 12 N NaOH) and adjusted to pH = 8. A methanolic NBD-Cl solution (0.1 ml, 25 µM/ml) is added and the reaction allowed to proceed in the dark at 40° C during one hour (3). Thereafter, the reaction mixture is adjusted to pH 3-4 by adding 0.2 ml 6 N HCl. The NBD-derivatives are then extracted with successively 3, 2 and 2 ml diethylether. The combined ether extracts are dried over sodiumsulfate and concentrated under a jet of nitrogen, according to the concentration range investigated, to a volume of 0.2-1 ml.

- Quantitative high performance thin layer chromatography (HPTLC)

The extracts are analysed by bi-dimensional HPTLC using silicagel 60 (aluminium sheets; first direction : methylenechloride : methanol 98:2 v/v; second direction : methylenechloride : propionic acid 98:2 v/v). After development and induction of the fluorescence with an alcaline cysteine solution (1), the relative fluorescence intensities of the thyreostatic drug derivatives are measured against the internal standard derivative (DMTU).

Results and discussion

- Mercuration of strong anion-exchangers

It was found that strong anion-exchangers (e.g. DOWEX 1) adsorb mercurial dyes (e.g. 2,7-dibromo-4-hydroxymercurifluorescein (DBMF)). Neither 0.5 N HCl nor 0.5 N NaOH are capable to strip off the dye from the resin. The binding characteristics of DBMF with DOWEX 1 resins of various cross-linkages were tested. DOWEX 1 x 2 was selected for clean-up of thyreostatic drugs; this resin bonds 25 mg DBMF/ml resin, equivalent to 6.7 mg (33 µM) Hg^{++}/ml wet resin.

- Study of adsorption and elution characteristics of thiouracil on DBMF columns

The adsorption of thiouracil on DBMF columns was studied. After washing the column with distilled water the drug was eluted with an acid salt (0.5 M NaCl) solution. The elution yields of TU, at different pH values, in function of the elution volume, are given in Fig. 3. A salt solution, with a pH value less than 1, results in an optimal elution of thiouracil from the column.

The adsorption and elution yields of TU and other thyreostatic drugs are summarized in Table 1. The adsorption of the drugs from a methanol-water (80:20, v/v) extract (10 ml) on micro-DBMF columns (0.6 ml) is practically quantitative. Through elution with 5 ml of 0.5 M NaCl (0.1 N HCl, pH = 1) most of the thyreostatic drugs studied (TU, MTU, PTU, DMTU) are recovered in a 80 % yield. On the contrarary, lower recoveries were noted for PhTU and TAP. The interaction of the phenyl group with the polystyrene matrix of the resin may explain the strong adsorption of PhTU. The small elution yield of TAP was due to a partial oxidation of the molecule on the column.

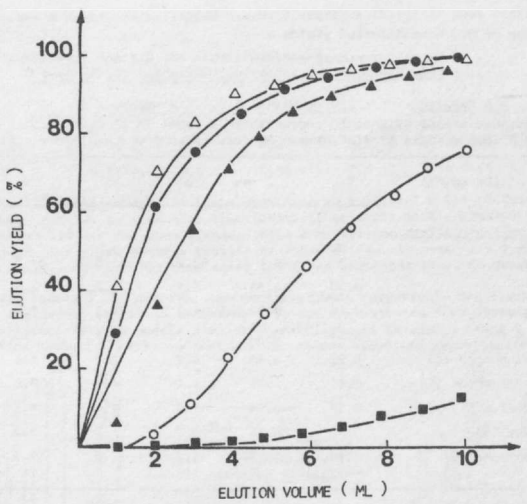


Fig. 3 : Elution of TU from a micro-DBMF column, at different pH values, in function of the eluens volume.
(■ pH = 4; ○ pH = 3; ▲ pH = 2; △ pH = 1; ● pH = 0.5)

Table 1 : Adsorption and elution yields of thyreostatic drugs on DBMF columns (column 0.4 x 5 cm).

thyreostatic drug		TU	MTU	PTU	DMTU	PhTU	TAP
not adsorbed (%)		3.8	0.5	1.5	0.5	8	0.5
elution yield : 5 ml (%)		82	80	78	79	17	60*
(pH = 1)	10 ml (%)	96	92	94	95	24	80*

(*) partially oxidised

- Reproducibility and Recovery

The reproducibility of the determination of TU in meat extracts is given in Table 2. The coefficient of variation of the total procedure amounted to 13 %. The recovery of MTU, TU and PTU, at the 100 ppb level, using DMTU as internal standard is given in Table 3. Recoveries for meat were quantitative for the drugs studied. Recoveries in plasma and milk were quantitative for MTU but appreciably lower for PTU and TU.

Table 2 : Reproducibility of the analysis of thyreostatic drugs in meat (TU).

step in procedure	n	mean volume ± SD	coefficient of variation (%)
column elution	26	81 ± 3.9	4.8
derivatisation	26	76 ± 5	6.6
HPTLC	22	88 ± 4.7	5.4
total procedure	22	55 ± 6.9	12.6

n : number of determinations

Table 3 : Recovery of the thyreostatic drugs in various media using the internal standard procedure.

biological material	concentration added (ppb)	concentration found ± SD PTU	MTU	TU
meat (3)[1]	100	106 ± 5.5	102 ± 13.7	97 ± 21.8
plasma (3)[1]	100	84 ± 6.2	98 ± 5.0	76 ± 5.7
milk (3)[1]	100	88 ± 6.9	105 ± 3.6	85 ± 8.0

[1] : number of determinations

- MTU concentrations in thyroid, kidney and muscular tissues of slaughter animals

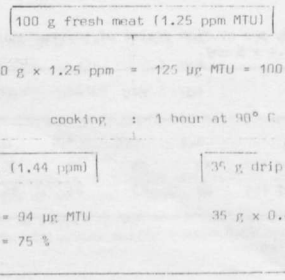
The concentration of MTU in the thyroid, the kidney and some muscles was determined in 5 animals, obtained from regulatory control. The results are summarized in Table 4. As expected, the highest concentration of MTU was found in the thyroid. The MTU concentration in the thyroid was 20-100 times the mean muscle concentration (0.2-1.9 ppm). The concentration of MTU in the kidney was significantly higher ($p \leq 0.005$) than the concentration found in muscular tissues. A significant difference ($p \leq 0.005$) in MTU content was found between the M. psoas (P), the M. diaphragma (D) and the M. trapesius (T) (non parametric test of Wilcoxon). Classification of the different tissues in descending order of MTU concentration yields :

thyroid > kidney > P = D = LD > N > S > G > T

Table 4 : Comparison of the MTU concentrations (ppm) in thyroid, kidney and some muscles of slaughtered animals taken from regulatory control.

number of the animal	1	2	3	4	5
tissue analysed					
thyroid	30.6	48	53.2	41.5	37.5
kidney	0.30	0.81	2.1	2.3	2.4
M. long dorsi (LD)	0.16	0.52	1.0	2.3	1.8
M. psoas (P)	0.21	0.63	1.7	2.3	1.3
cervical muscle (C)	0.22	0.40	1.2	1.7	1.5
M. gastrocnemius (G)	0.15	0.79	1.8	1.6	0.8
M. trapesius (T)	0.17	0.54	1.0	1.7	1.2
M. sorius (S)	0.18	0.46	3.0	1.7	1.2
M. diaphragma (D)	0.19	0.59	1.7	2.0	1.3

Fig. 4 : Effect of cooking on the residue concentration of MTU in meat.



- Effect of cooking on the MTU concentration of meat

Most of the meat consumed is prepared by heating. The fate of MTU during heating of meat was investigated using muscles of animals, taken from regulatory control at the slaughterhouses.
A meat cut was divided in two portions : one portion was analysed directly. The other portion was heated in a plastic bag at 90° C during one hour. The cooked meat and the drip were separated. All fractions were analysed for its MTU content. In total, 4 different muscles have been analysed using the procedure described.
Typical results are given in Fig. 4. Our measurements show that MTU is not appreciably destroyed in meat after prolonged heating. Since only 25 % of the total MTU content is recovered in the drip (= 35 % of the muscle weight), the MTU residues are concentrated in the cooked meat.

Conclusions

The use of a low cost mercurated adsorption column allows a selective and reproducible extraction of the thyreostatic drugs from samples of biological origin. This rapid clean-up procedure, coupled with the specific fluorescence detection after HPTLC-chromatography of the thiouracil derivates, permits a quantitative determination of these drugs at the ppb level. In comparison with earlier methods (1-3) the use of a mercurated resin increases the selectivity and speed of analysis : routinely more than 20 samples can be prepared for chrometographic analysis in less than one working day.
In cow carcasses, obtained from regulatory control, the residue levels in the thyroid were 20-100 times higher than in the corresponding muscular tissues. Cooking experiments demonstrated that MTU residues in meat are not appreciably destroyed by heating.

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