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A MODIFIED HPLC METHOD FOR THE DETECTION OF 6-METHYL-2-THIOURACIL IN CATTLE URINE

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Introduction

Thyreostatic drugs have been applied illegally to farm animals to get an increased live weight gain. This gain is due to a higher water retention in edible tissues and this results in a fraudulent overweight of meat due to the higher water content. Furthermore, thiouracils remain in meat with potential toxic consequences for consumers.

Treatment of cattle with thyreostatics drugs can be detected through its analysis in residues from organs, excreta, plasma, meat and urine of the animals. In the inspection control, urine and faeces, as well as plasma, of the animals may be sampled at the farm (Courtheyn et al., 2002). A number of methods for the detection, determination and confirmation of thyreostatic drugs in cattle urine have been previously reported (Pochard et al., 1984; Moretti et al., 1993; Buick et al., 1998; De Wasch et al., 2001). Recently, the criteria for the interpretation of test results of official control laboratories within the European Union has been regulated in the Decision 2002/657/EC (EC, 2002). The presented method has been validated according to this Decision.

Objectives

The goal of this work was the optimization and validation of a procedure for the detection of thyreostatic agents, specifically 6-methyl-2-thiouracil (MTU), in cattle urine through reverse-phase high performance liquid chromatography (RP-HPLC).

Methodology

The methodology has been based on the work of Moretti et al. (1993) developed for plasma with some modifications. A scheme of the extraction protocol is shown in figure 1. Briefly, 1 mL of cattle urine with added internal standard (5,6-dimethyl-2-thiouracil) were placed in a test tube. 100 mg EDTA disodium salt, 3 mL ethyl acetate and 15 µL mercaptoethanol. After vortexing and mixing for 10 min, the tube was frozen for 10 min, at least. The organic layer was transfered to a clean tube and evaporated under nitrogen. After resuspension in methanol, it was transfered to an HPLC vial and evaporated again. Then, the evaporated sample was resuspended in 200 µL of mobile phase consisting in 25 mM phosphate buffer, pH 3.0, with 10% (v/v) methanol. 10 µL were injected into an Agilent series 1100 HPLC equipped with a diode-array detector. The column was a Kromasil C18, 150 mm x 4.6 mm, from

Scharlau (Barcelona, Spain) with a flow rate of 1 mL per min. The gradient ranged from 10% methanol in phosphate buffer to 26% at 8 min; then, it increased until 70% in 2 min and remained at that percentage for 7 min. Afterwards the initial conditions were recovered in 3 min. The eluent was monitored at 276 nm. In general, the following order of injections was followed for each set of samples: i) reagent blank, ii) compliant (urine blank) control sample, iii) sample to be confirmed, iv) compliant control sample again and v) non-compliant (urine fortified with 150 ng mL^{-1} MTU) control sample.

Results & Discussion

Specificity: The method discriminated very well between the analyte (6-methyl-2-thiouracil) and closely related substances as can be appreciated in the chromatogram (see figure 2). As can be observed, potentially interfering substances (chemically related compounds) eluted at different retention times.

Recovery: The recovery was determined by experiments using a total of 90 fortified blank urine samples. Three sets, 30 samples each, were added 6-methyl-2-thiouracil to a final concentration of 100, 150 and 200 ng mL^{-1} for each set, respectively. The recoveries are shown in table 1. The mean recovery was 64.9%, equivalent to a recovery factor of 1.54.

Repeatability: A set of homogenized blank urine samples were fortified with 100, 150 and 200 ng mL^{-1} of 6-methyl-2-thiouracil. At each level, the analysis was performed with 6 replicates and the mean concentrations, standard deviations and coefficients of variation were determined (see table 2).

Within-laboratory reproducibility: A similar procedure as described above for repeteability was performed with different operators and under different environmental conditions. Results are shown in table 3.

Decision limit (CCα): 26 blank urine samples were analyzed and the signal to noise ratio calculated in the time frame where MTU is expected. The decision limit was set as 3 times the signal to noise ratio. This gives a $CC\alpha = 100$ ng mL^{-1}.

Detection capability (CCß): 34 blank urine samples were fortified with 100 ng mL^{-1} MTU, that corresponds to the decision limit, and the standard deviation was calculated. The value of the decision limit plus 1.64 times the standard deviation of the within-laboratory reproducibility of the measured content equals the detection capability. The obtained CCß was 130 ng mL^{-1}.

Conclusions

The RP-HPLC method for the analysis of 6-methyl-2-thiouracil in cattle urine has been validated using urine fortified at levels up to 600 ng mL^{-1}. The main recovery is about 65% (recovery factor of 1.54). The decision limit ($CC\alpha$) is 100 ng mL^{-1} and detection capability ($CC\beta$) is 130 ng ml^{-1} . Specificity, sensitivity and repeteability have been also validated using this protocol.

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Tables and Figures

Table 1.- Recovery

Level (ng mL^{-1})	**Mean recovery (%)**	**Standard deviation**	**CV (%)**
100	60.5	14.6	24.1
150	64.4	8.8	13.6
200	69.8	16.1	23.1
Mean	64.9	14.0	21.6

Table 2.- Repeteability

Level (ng mL^{-1})	**Mean recovery (%)**	**Standard deviation**	**CV (%)**
100	66.1	13.5	20.4
150	105.6	6.8	6.5
200	166.5	28.1	16.9

Table 3.- Within-laboratory reproducibility

Level (ng mL^{-1})	**Mean recovery (%)**	**Standard deviation**	**CV (%)**
100	56.5	14.4	25.4
150	94.0	18.7	19.9
200	121.6	20.3	16.7

Figure 1.- Scheme of the extraction protocol

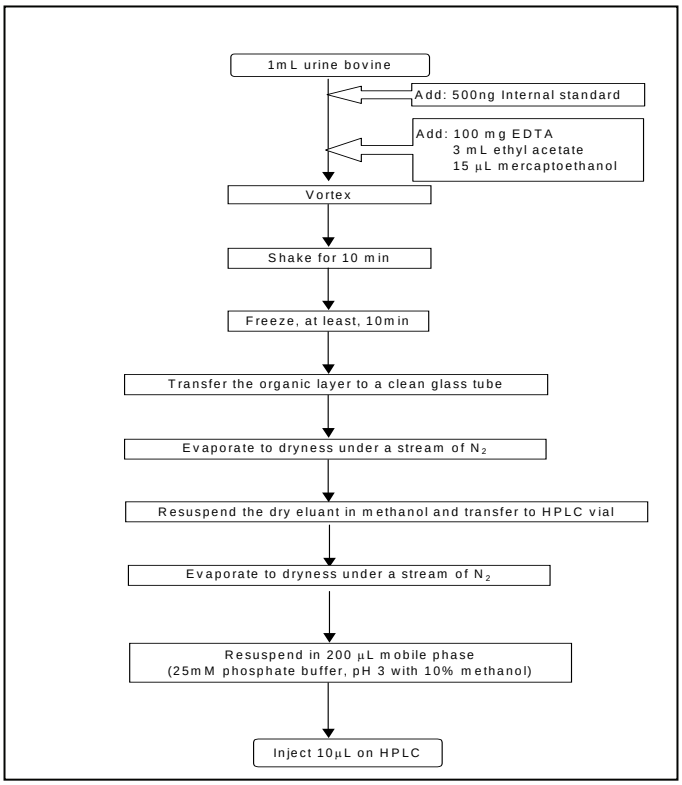

Figure 2.- Chromatogram of 6-methyl-2-thiouracil and closely related substances for the specificity study. TU: 2-thiouracil; MTU: 6-methyl-2-thiouracil; DMTU 5,6-dimethylthiouracil; PTU: 6-propyl-2-thiouracil.