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A FAST GC-MS METHOD FOR THE DETERMINATION OF TAPAZOLE IN BOVINE URINE

P. BATJOENS, H. F. DE BRABANDER and J. VAN HOOF

Dept. of Veterinary Food Inspection, Lab Chemical Analysis
Faculty of Veterinary Medicine of the University of Ghent,
Casinoplein 24 , B-9000 Ghent, Belgium

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INTRODUCTION

Thyreostatic drugs (TS) inhibit the function of the thyroid gland and cause a decreased production of thyroid hormones resulting in an abnormal increase of weight gain (Fox *et al.*, 1973). However, this weight gain consists mainly of an increased filling of the gastro-intestinal tract and augmented water retention in the slaughter animals (Aliev and Gasanov, 1980). The possibility of inferior meat quality and the potential that thyreostatic residues may be harmful for human health resulted in a ban of these substances as growth-promoters in all E.C.-member states (Owen *et al.*, 1973; EC, 1981; EC, 1986).

The most important and powerful thyreostatic drugs presently in use (De Brabander and Verbeke, 1984) are thiouracil (TU) and analogous compounds (especially methylthiouracil (MTU) and tapazole (TAP, Figure 1).

Extraction of TAP by means of the classical mercury column (2,7-dibromo-4-hydroxymercurifluorescein, DBMF) is not completely suitable for quantitative analysis, because of partial oxidation (De Brabander *et al.*, 1992).

Therefore it was the aim of this experiment to evaluate an alternative extraction method that is easy and fast to perform and compatible for quantitative analysis using GC-MS. The investigated extraction solvents were very specific for the extraction of TAP and not for the other thyreostatic drugs (Skellern *et al.*, 1976; Bending and Stevenson, 1978).

In a second part of this paper, the elimination of TAP was followed during the next two days using this fast extraction coupled to a quantitative determination by means of a Finnigan MAT Magnum Ion Trap System.

MATERIALS AND METHODS

Reagents and standard solutions

Chloroform (E. Merck, Darmstadt, Germany) and dichloromethane (E. Merck, Darmstadt, Germany) were used as possible extraction fluids. MSTFA (N-Methyl-N-trimethylsilyl-trifluoroacetamide) was obtained from Macherey-Nagel (Düren, G.F.R.).

Tapazole standard (1-Methyl-2-Mercaptoimidazole)(TAP) was purchased from Fluka Chemie (Bornem, Belgium). A stock solution of TAP was prepared in methanol at a concentration of 200ng/μl. A working solution (20ng/μl) was obtained by ten-fold dilution in methanol.

Analytical procedure

Extraction

Two ml of urine was put into a 100ml separation funnel and 5ml of chloroform or dichloromethane was added.

The two phases were manually shaken intensively for two minutes. Afterwards the two layers were let stand for 30 seconds to obtain optimal separation. The solvent layer was collected in a lab designed glass tube (Figure 2). This extraction procedure was repeated on the upper layer, consisting of the urine sample.

The combined extraction phase (±10ml) was dried completely in a Savant SpeedVac SC210A vacuum concentrator.

Derivatisation

The dry residue was derivatised with MSTFA (50μl) to form di-TMS derivatives (De Brabander *et al.*, 1992) and brought over to an autosampler vial.

Derivatisation of samples could be carried out in autosampler vials after transfer of the sample and further drying under a nitrogen stream.

Alternatively the special residue tubes could be used to avoid unnecessary transfers of the solvents (Figure 2). The diameter of these tubes (18mm) was chosen to fit the tube holders of the Speedvac. Other tube containing apparatuses (fraction collector of HPLC, sample preparation unit) were adapted to hold the same tubes. The length of the tube was adapted (shortened) to the syringe used for HPTLC spotting. These tubes have a maximum capacity of 17ml solvent (10ml in this application) but volumes as small as 20-30ul could be handled (e.g., derivatisation with 50μl MSTFA). These "all purpose" residue tubes could be used to collect relative large amounts of solvents (15ml), evaporate automatically and redissolve the analyze in small amounts (μl) of (another) solvent without tube transfer. This saves time.

Direct derivatisation for GC-MS could also be performed in the residue tubes. In that case a small reaction chamber is formed by introducing a glass rod covered at one side with teflon.

The external standard consisted of 4ng TAP/μl and was prepared by evaporation of 10μl of the TAP working solution (20ng/μl) in an autosampler vial, until complete dryness and derivatisation (15 minutes; at 60°C) with MSTFA (50μl).

GC-MS analysis

GC-MS analysis was performed with an Magnum Ion Trap Mass Spectrometer (Finnigan MAT, San Jose, CA, USA) fitted with a 25m x 0.20mm i.d. column coated with a 0.11μm film (HP ultra 2).

GC-MS conditions: initial column temperature: 100°C, to 200°C at 15°C/minute to 300°C at 30°C, ISO at 300°C during three minutes (total program ca 13 minutes). Injector temperature: 260°C, transfer-line: 300°C: carrier gas: helium.

Acquisition method: one scan/second during 10 minutes in a mass range of 80 to 400amu, filament multiplier delay: 300 seconds, ionisation by electron impact.

Sample collection

A three year-old cow was administered 1g TAP orally after collecting a urine sample was used as a reference sample. At regular intervals urine was sampled for analysis on TAP residues until 48 hours after administration. (urine samples at 3, 6, 9, 12, 24, 27, 30, 33, 36 and 48 hours). The urine samples were stored refrigerated at -18°C.

RESULTS AND DISCUSSION

Comparison of the two organic solvents as extraction fluid.
Based on data from literature (Skellern et al., 1976; Bending and Stevenson, 1978) the extraction yield of TAP was estimated using chloroform ($CHCl_3$) and another organic solvent (dichloromethane (DCM)).

The recovery of TAP was obtained by adding known, constant amounts of TAP to an urine blank (1000ng) and treating it in a similar manner as the unknown urine sample. One μl out of 50μl of the derivatised sample was injected into the GC-MS (20ng). The yield was calculated by comparison of the response after extraction of the spiked urine with DCM and $CHCl_3$ respectively with the response of 20ng TAP standard.

Quantitative analysis was performed using the external standard method, based on the area of the peak of the three combined diagnostic ions of TAP (171, 186, 259).

Figure 3A shows two chromatograms: the total ion chromatogram of TAP and the chromatogram of the combined diagnostic ions of TAP, obtained by extraction with $CHCl_3$. Figure 3B displays the typical electron impact spectrum of TAP.

The extraction yield obtained with the two solvents is summarized in Table 1.

The results in Table 1 show some variation within each solvent recovery, but $CHCl_3$ offered a better recovery (53 %) compared with the extraction with DCM (20%).

Therefore chloroform was chosen for the further experiment on the elimination of TAP in bovine urine.

Elimination of TAP in bovine urine after oral administration
Urine samples, collected after administration of 1g. TAP, were extracted with the above described $CHCl_3$ procedure and derivatised with MSTFA. One μl was injected into the GC-MS.

Figure 4 shows the elimination of TAP during the two days following the administration. Excretion was observed after three hours and reached a maximal excretion of about 441ppb after nine hours. Afterwards TAP residues decreased until 27 hours to about 76ppb. This first phase of elimination showed a linear decline (20.3ppb/hour). From 30 hours onwards, the excretion of TAP residues were a fifth of the rate in the first 27 hours (4.3ppb/hour). Unfortunately, the sampling was not carried out long enough to determine the end of the elimination. Thirty hours after administration, a little rise in the TAP residue concentration could be noticed (76ppb at 27 hours and 97ppb at 30 hours). This increase was probably due to the more concentrated urine sample. The analyses were carried out without correction for this effect. A determination of the creatinine clearance could solve this problem.

CONCLUSIONS

From the two studied organic solvents, chloroform and dichloromethane, the first offered the best recovery of about 53% compared with the extraction yield of only 20% for DCM. A recovery rate of 53% could not be considered efficient, but from the second part of this study on the elimination of TAP in bovine urine, this yield was sufficient for analyzing real urine samples and for estimating the elimination rate.

Elimination of TAP after oral ingestion of a single dose of 1g started rapidly after administration (+3 hours) and increased until NINE hours. From this maximum concentration, a linear decline occurred until 27 hours. The following elimination rate decreased to about a fifth of the rate in the first 27 hours.

Unfortunately, the sampling was not carried out long enough to determine the end of the elimination.

Nevertheless, it can be concluded that this $CHCl_3$ extraction is a fast and easy to perform method, compatible for GC-MS analysis of TAP residues in bovine urine down to even less than 50ppb.

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Table 1. Extraction yield of TAP from urine, extracted with DCM and $CHCl_3$.

	Mean value (ng)	Yield (%)
20ng TAP standard	20	100
Spiked urine, DCM extraction	4.07 ± 0.26	20
Spike urine, $CHCl_3$ extraction	10.65 ± 1.46	53