

Rapid and high-performance analysis of thyreostatic drug residues in urine using gas chromatography–mass spectrometry

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Abstract

A more sensitive method was developed using the hyphenated technique of gas chromatography–mass spectrometry (GC–MS) supplementary to the official high-performance thin-layer chromatography (HPTLC) method. Even combined with less efficient extraction and clean-up methods, GC–MS is able to lower the detection limit to less than 50 ppb. The powerful technique of GC–MS–MS is tried out to reduce the detection limit even more, in combination with simplified extraction methods. This time-saving approach combined with the increase in sensitivity is of great importance for a routine technique.

Keywords: Thyreostatic drugs; Thiouracil; Methylthiouracil; Tapazole

1. Introduction

Thyreostatic drugs inhibit thyroid function; the decreased production of thyroid hormones reduces basal metabolism, lowers gastro-intestinal motility and favors extracellular water retention. The use of these substances thus allows a considerable increase in live mass gain, although this mainly results from an increased filling of the gastro-intestinal tract and augmented water retention in the slaughter animals [1–3].

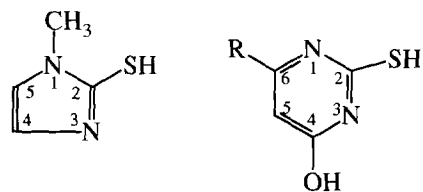
In contrast with anabolic agents, there is a general agreement in the EU on the ban of these drugs: Thyreostatic drugs may be harmful for human health and the meat derived from animals treated with the drugs may be of inferior quality. In Belgium, regulatory control on the illicit application of growth promoters is coordinated by the Institute of Veterinary Inspection (IVK-IEV) (Ministry of Human

Health) and the Ministry of Agriculture according to the EEC directives [4,5].

The most important and powerful thyreostatic drugs, hitherto used are thiouracil and analogous compounds (especially methylthiouracil (MTU) and tapazole (TAP); see Fig. 1).

Specific detection procedures for the detection of this group of drugs has been described previously [6–8].

Chromatographic techniques have the advantage of not only detecting abuse of thyreostatic drugs but



I: 1-methyl-2-mercaptoimidazole (tapazole)

II: 4(6)-R-2-thiouracil (R = H, methyl, n-propyl or phenyl)

Fig. 1. Structural formulae of thyreostatic drugs.

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are also capable of identifying the different molecules or groups of substances.

HPTLC is used as the official method for the qualitative and quantitative determination of residues of thyreostatic drugs in biological material. The method is based on the fluorescence induction of the NBD (7-chloro-4-nitrobenzo-2-oxa-1,3-diazole)-derivatives of the drugs with cysteine after HPTLC separation. The clean-up is performed with a rapid and selective extraction procedure, based on specific complex formation of the drugs with mercury ions bound in an affinity column. These methods have been adopted by the Benelux countries [9] and the EEC member states [10].

Schilt et al. [11] also used the mercurated affinity column for sample clean-up in combination with a GC-NPD method.

The HPTLC method may also be coupled to additional confirmation of the suspect "spots" by GC-MS with positive chemical ionisation [12].

The abuse of these substances could be drastically reduced by regulatory control. Many years passed without any observation of thyreostatic residues.

Moreover, since the control of anabolic agents was intensified during the last few years, thyreostatic drugs risk being used again. Over the last few years routine analysis of some fodder samples revealed the presence of tapazole. Urine samples and thyroid gland samples, however, were mostly free of tapazole residues using HPTLC. Therefore, a more sensitive method was developed using the hypenated technique of GC-MS. Thyreostatic drugs are easily detected by GC-MS with positive chemical ionisation and electron impact.

Besides the confirmation of the suspect spots on HPTLC by GC-MS and now even GC-MS-MS, a rapid extraction method specific for tapazole is evaluated using the power of tandem MS (MS2).

2. Experimental

2.1. GC-MS apparatus and conditions

GC-MS analysis was performed with a Magnum Ion Trap Mass Spectrometer (Finnigan MAT, San Jose, CA, USA) consisting of a Magnum Ion Trap System (Finnigan MAT) consisting of a Finnigan

MAT A200S GC Autosampler, a Varian 3400 GC with 1077 capillary split/splitless injector, a Finnigan MAT Magnum Ion Trap Mass Spectrometer with electron impact and advanced positive chemical ionisation and fitted with a 25 m×0.20 mm I.D. column coated with a 0.11 μm film (HP Ultra 2).

GC-MS conditions: Initial column temperature: 100°C, to 200°C at 15°C/min, to 300°C at 30°C/min, ISO at 300°C for 3 min (total program ca. 13 min). Injector temperature, 260°C; transfer-line, 300°C; carrier gas, helium.

Acquisition method: 1 scan/s over 10 min in a mass range of 80 to 400 a.m.u.; filament multiplier delay, 300 s; ionisation by electron impact.

GC-MS-MS was carried out with the GCQ (Finnigan MAT) consisting of a Finnigan MAT A200S GC Autosampler, a Finnigan MAT/Tremetrics high-performance capillary GC, a capillary split/splitless injector with electronic pressure control and a Finnigan MAT Quadrupole Ion Trap Mass Analyzer.

GC-MS conditions: GC temperature program, identical to that of the Magnum. Column, SGE BPX-5 (25 m×0.22 mm I.D., film thickness 0.25 μm).

MS mode: cfr. Magnum.

Tandem MS mode: 1 scan/s, mass range from 50 to a mass that was 1 unit higher than that of the parent ion selected (the parent ion is mostly the base peak of the full scan spectrum of the molecule); collision energy for fragmentation of the parent ion, 0.7–3.0 V.

2.2. Reagents and standard solutions

2.2.1. Reagents

Chloroform and dichloromethane (E. Merck, Darmstadt, Germany) were used as possible extraction fluids. MSTFA (N-methyl-N-trimethylsilyl-trifluoroacetamide) is from Macherey-Nagel (Düren, Germany).

2.2.2. Standard solutions

Stock solution of the thyreostatic drugs (TU, thiouracil; MTU, methylthiouracil; PTU, propionylthiouracil and TAP, tapazole) (Fluka Chemie, Bornem, Belgium) were prepared in methanol at a concentration of 20 mg/100 ml (200 ng/ μl). A

working solution is obtained by $100\times$ dilution in methanol ($2\text{ ng}/\mu\text{l}$).

Internal standard solution: Dissolve 20 mg of DMTU (4(5,6)-dimethyl-2-thiouracil) in 100 ml of methanol ($200\text{ ng}/\mu\text{l}$). A working solution is obtained by $100\times$ dilution in methanol ($2\text{ ng}/\mu\text{l}$).

2.3. Analytical procedures

2.3.1. Extraction method for all thyreostatic drugs in urine

A 2-ml volume of urine was mixed with 10 ml of methanol. An amount equivalent to 100 ppb of DMTU was added as the internal standard and the solution was allowed to percolate through a mercury column. After washing with distilled water, the thyreostatic drugs were eluted with a solution of hydrochloric acid (0.1 M) in a sodium chloride solution (0.5 M, 5 ml, pH 1). Buffer solution (pH 8, 1 ml) is added to the eluate. The eluate was then neutralized and was, after adjusting to pH 8, derivatized with a NBD-Cl (7-chloro-4-nitrobenzo-2-oxa-1,3-diazole) solution. The formed NBD-derivatives were extracted with diethyl ether and the concentrated extract (by evaporation) was spotted on the HPTLC plate.

2.3.2. Extraction method for tapazole in urine

A 2-ml volume of urine was placed in a 100-ml separation funnel and 5 ml of chloroform or dichloromethane were added.

The two phases were manually shaken intensively for 2 min. Afterwards, the two layers were left standing for 30 s to obtain optimal separation. The solvent layer was collected in a laboratory-designed glass tube (Fig. 2). This extraction procedure was repeated on the upper layer, consisting of the urine sample.

The combined extraction phase ($\pm 10\text{ ml}$) was

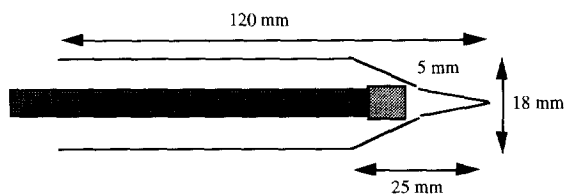


Fig. 2. Laboratory-designed special residue tube.

dried completely in a Savant SpeedVac SC210A vacuum concentrator.

2.3.3. Derivatisation

The dry residue was derivatised with MSTFA ($50\mu\text{l}$) to form mono-TMS derivatives [12] and was then placed in 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 transfer of solvents (Fig. 2). The diameter of these tubes (18 mm) was chosen to fit the tube holders of the Speedvac. Other apparatus used for containing tubes (fraction collector of HPLC, sample preparation unit) were adapted to hold the same tubes. The length of the tube was adapted (shortened) to the syringes used for HPTLC spotting. These tubes have a maximum capacity of 17 ml solvent (10 ml in this application) but volumes as small as $20\text{--}30\mu\text{l}$ could be handled (e.g. derivatisation with $50\mu\text{l}$ MSTFA). These "all purpose" residue tubes could be used to collect relatively large amounts of solvents (15 ml), evaporate them automatically and redissolve the analyte in small amounts (μl) of (another) solvent without tube transfer. This saves a lot of time.

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

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

3. Results and discussion

3.1. GC-MS detection of thyreostatic drugs

Different reagents were possible as the derivatisation agent for thyreostatic drugs. MSTFA was tried first because it was also used for the derivatisation of steroids. It was found that the most important TS could easily be derivatised with MSTFA to mono-TMS (TAP) and di-TMS (TU, MTU, DMTU and

PTU) derivatives. These derivatives could be separated on an apolar column (e.g. HP Ultra 2, SGE BPX-5). This was very important for the routine operation of the GC–MS because the MSTFA derivatives could be injected immediately, without evaporation and redissolving in any solvent. So the column that was used for other purposes (with MSTFA) could be used. As can be seen from Table 1, the five thyreostatic drugs of importance are all well chromatographed under these conditions with retention times varying between 5 and 8 min. All the mass spectra show at least two characteristic ions. These ions are listed in Table 1.

Therefore GC–MS–MS could be the solution to obtain more diagnostic ions to increase the specificity of the detection. The MS2 spectra were obtained by colliding the base peak of the MS spectrum which resulted in three usable product ions. The MS2 spectra in EI mostly contained two to three ions in the lower mass range. For the five different molecules, only two different base peaks were obtained i.e. 99 for TU, MTU and PTU and 113 for TAP and DMTU.

The intensities of the different ions varied for the different molecules, but the diagnostic ions were practically identical for the two groups.

Table 1
Retention times and diagnostic ions of thyreostatic drugs in electron impact (MS and MS2)

	Retention time ^a	Diagnostic ions	
		Electron impact	Electron impact MS2
TU	5:53	257	99
		99	147
		272	75
TAP	5:55	171	113
		186	130
		113	171
MTU	6:18	271	99
		99	149
		285	75
DMTU	6:55	285	113
		113	75
		299	147
PTU	7:23	299	99
		99	147
		149	75

^a Indicative.

Table 2

Extraction yield of TAP from urine extracted with DCM and CHCl₃

	Mean value (ng)	Yield (%)
20 ng of TAP standard	20	100
Spiked urine, DCM extraction	4.07±0.26	20
Spiked urine, CHCl ₃ extraction	10.65±1.46	53

3.2. Comparison of the two organic solvents as extraction fluids for fast determination of tapazole

Based on data from literature [13,14], the extraction yield of TAP was estimated using chloroform (CHCl₃) and another organic solvent [dichloromethane (DCM)].

The recovery of TAP was obtained by adding well-known constant amounts of TAP to blank urine (1000 ng) and treating them in a similar manner to that of the unknown urine samples. A 1- μ l volume of the 50 μ l derivatised sample was injected into the GC–MS (20 ng). The yield was calculated by comparison of the response after extraction of the spiked urine with DCM and CHCl₃, respectively with the response of 20 ng of 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, 113).

The extraction yield obtained with the two solvents is summarized in Table 2.

The results in Table 2 show some variation within each solvent recovery, but CHCl₃ offered a better

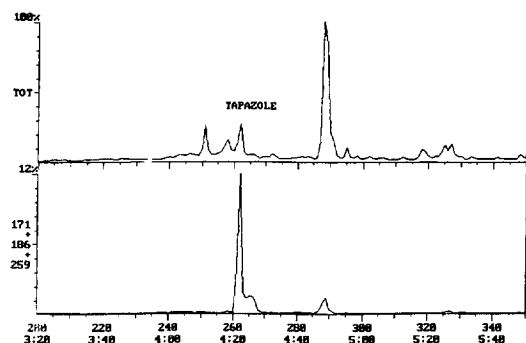


Fig. 3. Chromatogram of tapazole residues (440 ppb) in urine, 9 h after administration of 1 g of tapazole.

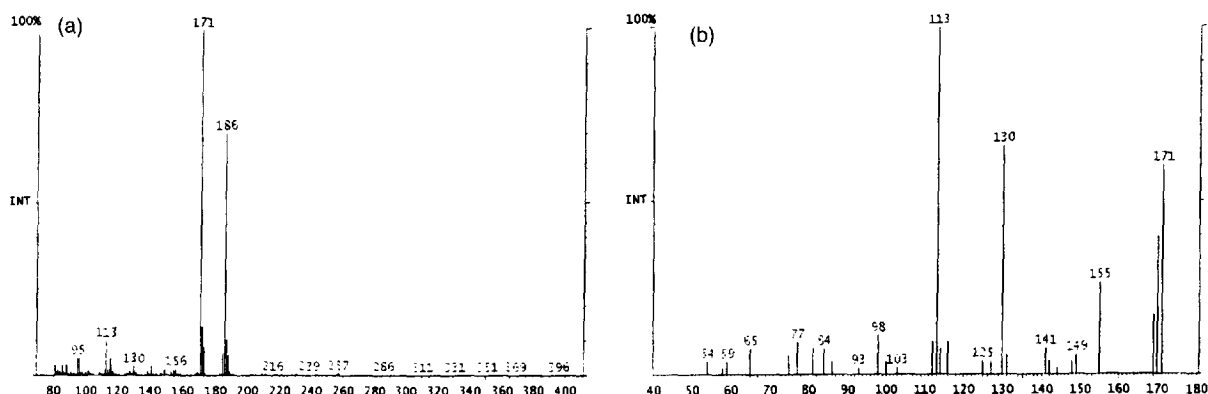


Fig. 4. Electron impact spectra of tapazole in a spiked sample (500 ppb) [MS (a) and MS2 (b)].

recovery (53%) than the extraction with DCM (20%) did.

Therefore, chloroform was chosen for the further experiment on the performance of the GCQ as a rapid detection method for TAP in bovine urine.

Fig. 3 presents a chromatogram of tapazole residues in a urine sample 9 h after administration of 1 g of tapazole.

3.3. Tandem MS for tapazole detection in urine

Urine samples spiked at 500, 250, 100 and 50 ppb were extracted with chloroform according to the method described before. Decreasing the amount to 100 ppb, this simple method was able to detect tapazole residues at two or three diagnostic ions in MS or tandem MS, respectively. Fig. 4 shows the EI-spectra of tapazole in MS and MS2 (standard and urine sample spiked at 500 ppb).

The combination of the rapid extraction method for tapazole and the use of tandem MS for detection resulted in a higher specificity.

On the other hand, no improvement of sensitivity was achieved, as would normally be expected in tandem MS.

4. Conclusion

From the two studied organic solvents, chloroform and dichloromethane, the first offered the best re-

covery, of about 53%, compared with an extraction yield of only 20% for DCM. A recovery rate of 53% could hardly be called splendid, but compared with the sensitivity of the HPTLC method for tapazole (100 ppb) this very simple and rapid extraction method can be used in the analysis of urine samples as a supplementary method to the official HPTLC method.

The theoretical gain of sensitivity in tandem MS–MS, as seen for the analysis of anabolic steroids, is not accomplished for the analysis of thyreostatic drugs. Tandem MS–MS results from the analysis of thyreostatic drugs are situated in the lower mass ranges and are therefore less specific. Besides this, there is also the loss of specificity between the different molecules (only two different base peaks).

Another remarkable finding was the lack of reproducibility in the MS–MS spectra. Between different analyses, different settings of the collision energy were needed to obtain comparable daughter spectra.

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 100 ppb.

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