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SCREENING AND CONFIRMATION OF THYREOSTATICS IN URINE BY GAS CHROMATOGRAPHY WITH NITROGEN–PHOSPHORUS DETECTION AND GAS CHROMATOGRAPHY–MASS SPECTROMETRY AFTER SAMPLE CLEAN-UP WITH A MERCURATED AFFINITY COLUMN

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SUMMARY

Methods are described for the screening and confirmation of residues of the thyreostatics thiouracil, methylthiouracil and propylthiouracil in urine samples of cattle at levels down to 25 μg/l. After a selective preconcentration of the thiol-containing thyreostatics on a mercurated affinity column, the analytes are derivatized by extractive alkylation and analysed by gas chromatography with nitrogen–phosphorus or mass spectrometric detection.

INTRODUCTION

Substances that can inhibit the production of the thyroidal hormone thyroxine, thyreostatics, can be used to increase the slaughter weight of animals in livestock breeding. Owing to Dutch regulations and EC directive 86/469 [1], which bans the use of any drug to improve growth, a control programme to detect the possible use of anabolics, e.g. anabolic steroids and thyreostatics, has to be executed in all EC member states.

The thyreostatics, selected for screening in this programme, can be divided into two groups. The first contains thiouracil analogues, such as thiouracil (TU), methylthiouracil (MTU), propylthiouracil (PTU) and phenylthiouracil (PhTU). The second group consists of the mercaptoimidazole analogues, such as tapazol (TAP). These thyreostatics can be administered orally by mixing with feed or dissolving in drinking water. The substances are excreted in urine both as (unchanged) free and conjugated drugs [2]. Excretion studies in cows showed that

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MTU can be detected as a free unchanged compound, for several weeks after oral application [2–4].

For screening purposes a high-performance liquid chromatographic method for MTU has been developed in our laboratory using electrochemical detection [5]. For screening of TU, MTU, PTU, PhTU and TAP, a thin-layer chromatographic (TLC) method has been developed by De Brabander [2] and Verbeke and De Brabander [6,7]. In this method, high-performance thin-layer chromatography (HPTLC) with sensitive fluorescence detection is combined with a sample clean-up based on affinity chromatography. Using the interaction between thiol groups present in the analytes, and immobilized organic mercury molecules, the thyreostatics are selectively retained and a high degree of sample clean-up is obtained. Most of the analytes mentioned before are extracted with high efficiency (more than 90%), except for TAP and PhTU (less than 50%). An advantage of using the affinity column is the possibility of omitting an extraction of the thyreostatics from urine samples. Owing to their high polarity, such an extraction is only possible with very polar solvents. However, recoveries are low and a high level of impurities is obtained.

In our experience, the HPTLC method is excellent, although rather laborious, and it demands much experience for the evaluation of the HPTLC plates. To avoid false-positive results, the identity of the thyreostatics found with such a screening method has to be confirmed with a highly specific spectrometric method, such as gas chromatography–mass spectrometry (GC–MS). Prior to GC, the polar analytes have to be derivatized, and several procedures have been described [8,9]. Owing to the difficulty of extracting the analytes from aqueous solutions, a procedure using extractive alkylation was chosen.

This paper demonstrates the application of GC with nitrogen–phosphorus detection (NPD) for screening purposes and of GC–MS as a confirmation technique, combined with affinity chromatography as a selective sample clean-up, for the determination and confirmation of thyreostatics, with special attention to MTU, in urine samples from cattle.

EXPERIMENTAL

Chemicals

Tetrabutylammonium hydrogensulphate (TBAHS), dibromohydroxymercuryfluorescein (merbromin) and iodomethane were obtained from Merck (Darmstadt, F.R.G.). Dowex resin 1-X2, mesh size 50–100 μm, was obtained from Serva (Heidelberg, F.R.G.). The pure standards TAP, MTU, PTU and TU were obtained from Fluka (Buchs, Switzerland). The internal standard dimethylthiouracil (DMTU) was a gift from Dr. H. De Brabander (University of Ghent, Faculty of Veterinary Medicine, Ghent, Belgium). Solutions of the internal standard and the standards were prepared according to Verbeke and De Brabander [6]. Demineralized water, prepared with a Milli-Q system (Millipore, Bedford, MA, U.S.A.) was used. All other chemicals were of analytical-grade quality.

The elution solvent consisted of a solution of 29.2 g of sodium chloride in 1000 ml of 0.1 *M* hydrochloric acid.

The mercurated affinity material was prepared according to Verbeke and De Brabander [6] with minor modifications. A 40-ml volume of the Dowex resin, dispersed in water, was placed in a glass filter (pore size 40–100 μm) and successively washed with 400 ml of water, 400 ml of 0.5 *M* sodium hydroxide, 400 ml of water, 400 ml of 0.5 *M* acetic acid and 400 ml of water. The resin was transferred to a glass flask, and 200 ml of water, containing 1.0 g of merbromin, were added. After shaking for 4 h, the resin was successively washed in a glass filter (pore size 40–100 μm) with 400 ml of elution solvent, 1000 ml of water and 400 ml of 0.1 *M* sodium hydroxide. Finally the resin was washed with water until the pH value of the eluate was 7. The mercurated resin could be used for two months when stored in the dark at room temperature.

Just before use, 0.6 ml of the mercurated affinity material was packed in a column [57 mm×5.5 mm I.D. (No. 7121-01); J.T. Baker, Phillipsburg, NJ, U.S.A.] with a 20-μm frit on the bottom. A wet glass wool plug was placed on top of the material.

Equipment

The GC–NPD system consisted of a Carlo-Erba (Milan, Italy) Model 4160 gas chromatograph and a Model 40 nitrogen–phosphorus detector. The detector and injector temperatures were set at 280 and 230°C, respectively. A CP-Sil-8 CB column (fused-silica, open tubular, cross-linked, 25 m×0.25 mm I.D.; Chrompack, Middelburg, The Netherlands) with a film thickness of 0.12 μm was used, with helium as carrier gas. The temperature of the GC oven was programmed to rise from 80 to 105°C at 5°C/min, followed by a rise to 270°C at 10°C/min.

The GC–MS system (Hewlett-Packard, Rockville, MD, U.S.A.) consisted of a Model 5890 gas chromatograph, a Model 5970 mass selective detector and a Model 7673-A autoinjector. A CP-Sil-5 CB column (fused-silica, open tubular, cross-linked, 25 m×0.25 mm I.D.; Chrompack) with a film thickness of 0.12 μm was used, with helium as carrier gas. The temperature of the GC oven was programmed to rise from 80 to 290°C at 15°C/min.

Both GC systems were operated in splitless mode; the splitters were opened 2 min after injection.

The derivatization was performed in a Vortex-Evaporator heating–stirring device (Buchler, Braunschweig, F.R.G.).

Sample materials

A blank urine sample, taken from a male calf (twenty weeks old), was obtained from the Institute for Livestock Feeding and Nutrition Research (IVVO, Lelystad, The Netherlands). An MTU-containing lyophilized urine quality control and candidate reference sample (Code No. 26) was obtained from the Laboratory for Residue Analysis [National Institute of Public Health and Environmental Protection (RIVM), Bilthoven, The Netherlands]. The latter sample was prepared by 1000-fold dilution of a urine sample, taken from a steer, which had been orally treated with high doses of MTU for several weeks, with urine of non-treated steers.

Sample preparation

To one volume of urine sample (2–10 ml), five volumes of methanol and an adequate amount of the internal standard solution were added. After mixing for 10 s, the solution was centrifuged at 10 000 *g* for 10 min. The supernatant was applied to the mercurated affinity column, and the eluate was discarded. The column was washed twice with 5 ml of water. Subsequently the analytes were eluted with 5.0 ml of elution solvent into a 25-ml test-tube, which could be closed with a screw-cap with a PTFE insert.

Derivatization

To the eluate obtained from the mercurated affinity column, 2 ml of TBAHS solution (0.24 *M* in 0.95 *M* sodium hydroxide), 2 ml of dichloromethane and 200 μl of iodomethane were added. The tube was closed, and the mixture was simultaneously heated at 60 °C and intensively mixed for 30 min, using a heating–stirring device. After cooling, phase separation was enhanced by centrifugation (5000 *g*) for 5 min. The dichloromethane layer was isolated and dried over anhydrous sodium sulphate. After slow evaporation at 35 °C under a mild stream of nitrogen, the derivatives were dissolved in 500 μl of ethyl acetate by ultrasonification. After centrifugation for 5 min at 5000 *g*, the supernatant was separated from the precipitate and transferred to a vial with a screw cap and a PTFE insert.

For GC–NPD analysis the injection volume was 1 μl, and for GC–MS analysis it was 5 μl.

RESULTS AND DISCUSSION

As was shown in our laboratory, the HPTLC method [6], although not developed for the analysis of urine originally, had a limit of detection for the thiouracil analogues and TAP in urine of 50 and 100 μg/l, respectively. However, owing to interfering compounds, the evaluation of the HPTLC plates was difficult at this level. To avoid false-positive results, all extracts were spotted on two HPTLC plates: plate A, with an aliquot of the sample extract, and plate B, with a mixture of the sample extract and a combination of standards (co-chromatography). In our hands the HPTLC method was rather laborious and demanded much experience for the evaluation, in order to avoid false-positive results.

Gas chromatography with nitrogen–phosphorus detection

To provide a less difficult evaluation, a GC method was developed. The presence of nitrogen atoms in the thyreostatics provides the opportunity to use a nitrogen–phosphorus detector for screening purposes. However, derivatization is necessary for GC analysis, to decrease the polarity of the analytes. A derivatization procedure often used for these types of compound is alkylation [8,9]. Most of the alkylation procedures are performed in non-aqueous media, such as acetone or dichloromethane. So, prior to the derivatization, the analytes have to be extracted from the aqueous matrices. However, a typical and special problem with thyreostatics, and especially with the thiouracil analogues, is the high polarity of these analytes, which causes low extraction yields. Only solvent mixtures with a

high polarity, such as chloroform–ethyl acetate or chloroform–methanol, can be used. In our experience, the extraction efficiencies were low and many interfering substances were coextracted with these types of mixtures.

For this reason, the technique of extractive alkylation, as used for example in the analysis of TAP in plasma [9] and the analysis of highly polar diuretics [10], was introduced. Here, the polar compounds were ionized by adding an alkaline solution. The ionized compounds were extracted with a counter-ion (tetrabutylammonium) into an organic solvent (dichloromethane) and derivatized with an alkyl halide (iodomethane). During methylation the thiouracil analogues formed two derivatives with a intensity ratio of ca. 1:10 (Fig. 1), probably caused by the methylation of different groups in the molecules. For TAP, only a monomethyl derivative (on the sulphur atom) is possible.

As shown in Fig. 1A and B, a large unidentified peak, with variable shape and area, eluted between 9 and 11 min. By optimizing the temperature programme of

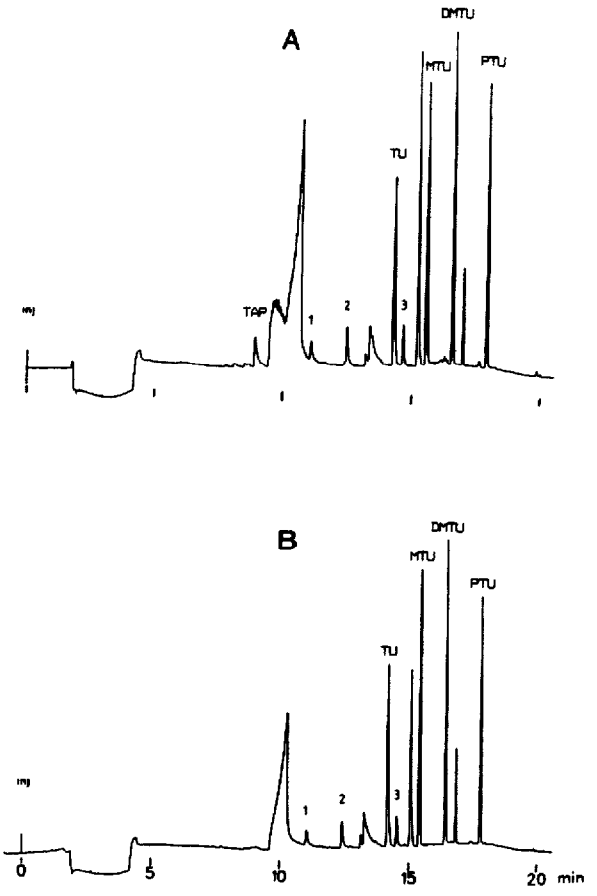

Fig. 1. Chromatograms (GC–NPD) of a water sample (4 ml) containing 1 mg/l TAP, TU, MTU, PTU and DMTU (attenuation 8); injection volume, 1 μl. (A) Direct extractive alkylation without clean-up. (B) Extractive alkylation after clean-up with the mercurated affinity column. Peaks 1, 2 and 3 indicate the side-product of TU, MTU and PTU, respectively.

the chromatograph, a baseline separation between this peak and the TAP peak was obtained. However, the extractive methylation of TAP had a low yield, resulting in a dramatic loss of sensitivity (Fig. 1A). Also, the extraction efficiency of the affinity column for TAP was low, which, together with the poor derivatization yield, resulted in the disappearance of TAP from the chromatogram (Fig. 1B).

Using the affinity column, the recovery of the thiouracil analogues TU, MTU and PTU from a standard solution was $95 \pm 3\%$ ($n=3$). Applying extractive alkylation only, the chromatogram of a blank urine sample, spiked with the thio-

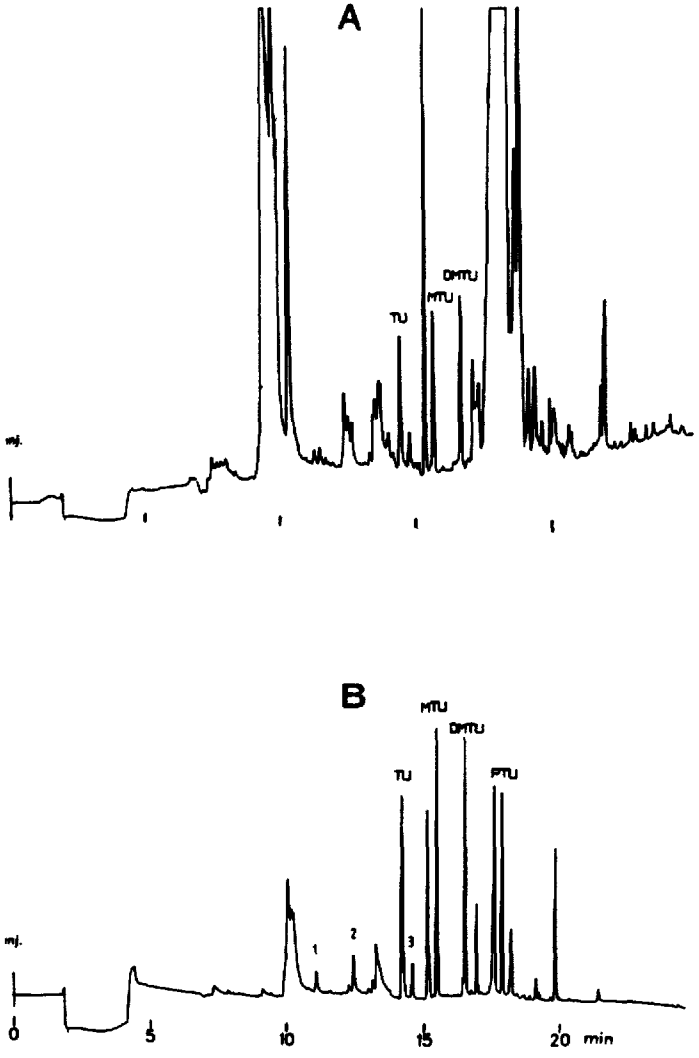

Fig. 2. Chromatograms (GC–NPD) of a blank urine sample (4 ml) spiked with TU, MTU, PTU and DMTU at 1 mg/l (attenuation 8); injection volume, 1 μl. (A) Direct extractive alkylation without clean-up. (B) Extractive alkylation after clean-up with the mercurated affinity column. Peaks 1, 2 and 3 indicate the side-products of TU, MTU and PTU, respectively.

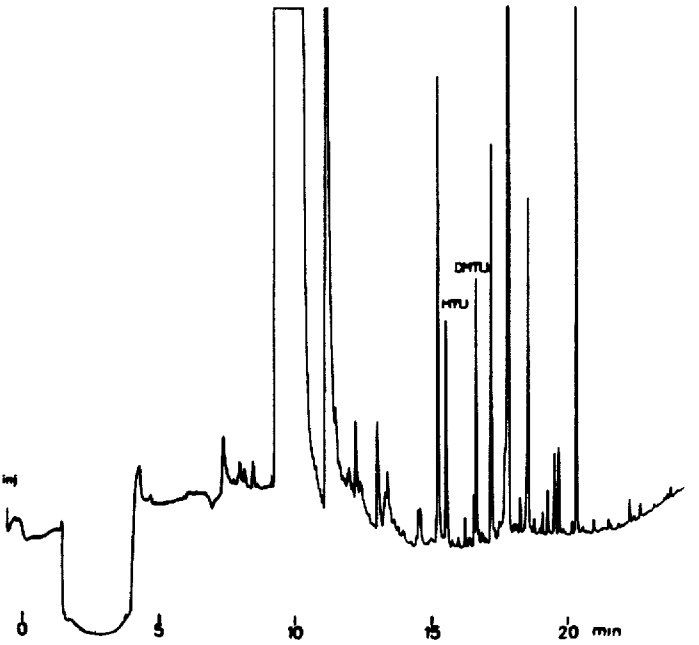

Fig. 3. Chromatogram (GC–NPD) of the MTU-containing quality control sample (2 ml) after clean-up with the mercurated affinity column (attenuation 2); injection volume, 1 μl. The internal standard (DMTU) was added at the 100 μg/l level.

uracil analogues at 1 mg/l, was moderately clear in the region where TU, MTU and DMTU (internal standard) elute (Fig. 2A). However, the detection of PTU was disturbed by interfering compounds. So, without the mercurated affinity column, the detection of the thiouracil analogues, except for PTU, is ca. 250 μg/l. Introducing the mercurated affinity column for sample clean-up, the chromatogram showed few peaks (Fig. 2B), and the thiouracil analogues could be identified without any problems below the 100 μg/l level. Increasing the sample volume from 4 to 10 ml, the detection limit could be lowered to ca. 25 μg/l.

Fig. 3 shows the chromatogram of the MTU-containing urine sample (quality control material). The concentration of MTU in this sample was calculated as 60 μg/l, which corresponds to the result obtained in our laboratory using the HPTLC method [6] and the indicated level given by RIVM.

Although not tested on many urine samples yet, the method using GC–NPD combined with affinity chromatography looks very promising for screening purposes. An advantage of this technique is the very short overall time needed for the analysis (ca. 2 h) and the easy evaluation of the chromatograms.

Gas chromatography–mass spectrometry

By using the same extraction and derivatization procedures as described for the GC–NPD screening, the thiouracil analogues can be detected selectively by applying MS. Although initially meant for confirmation purposes, this technique

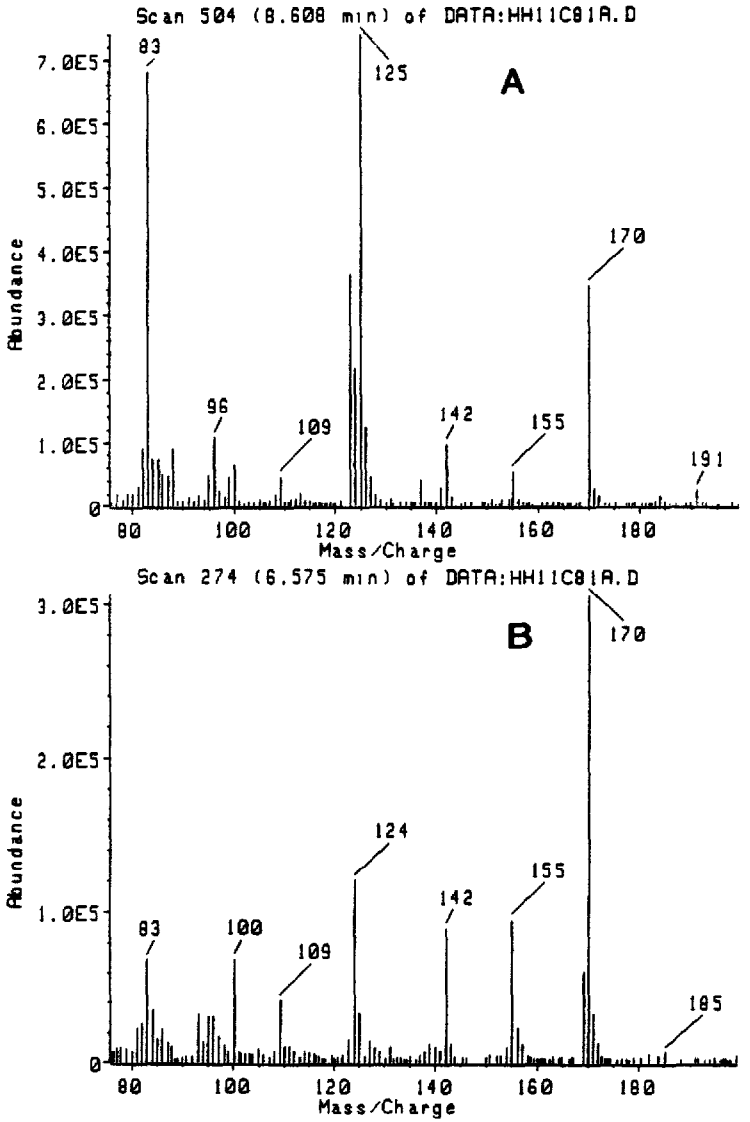

Fig. 4. Mass spectra of the two isomeric dimethyl derivatives of MTU. (A) Mass spectrum of the derivative with the higher yield. (B) Mass spectrum of the derivative with the lower yield, shown as peak 2 in Figs. 1 and 2.

can be applied for screening purposes when using, for instance, the GC–MSD (Hewlett Packard) equipped with an autoinjector.

The mass spectra of the two isomeric derivatives of MTU are shown in Fig. 4. For both compounds, the molecular ion had a mass of *m/e* 170, indicating the formation of a dimethyl derivative (*m/e* 142 + two methyl groups of *m/e* 14). The molecular ions found for PTU (*m/e* 198), DMTU (*m/e* 184) and TU (*m/e* 156) also indicated the formation of dimethyl derivatives. The results from a blank urine sample, spiked at the 1 mg/l level and detected in full-scan mode, are shown

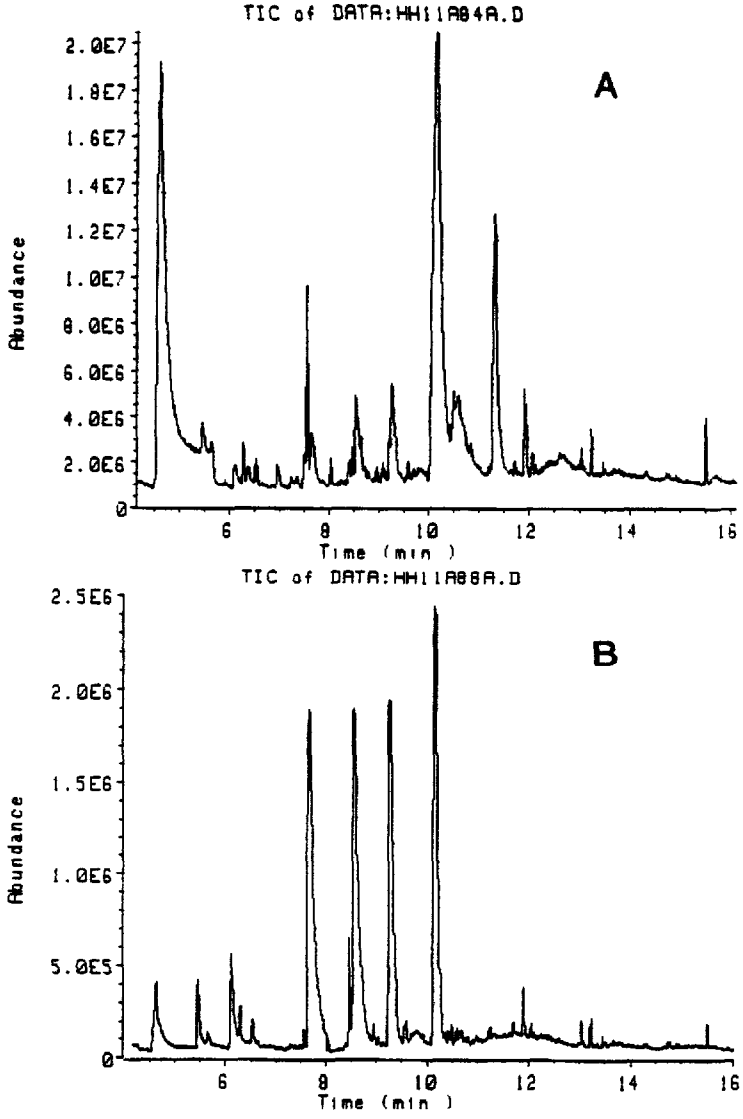

Fig. 5. Total ion chromatograms (GC–MS) of a blank urine sample (4 ml) spiked with TU (retention time 7.66 min), MTU (retention time 8.56 min), PTU (retention time 10.15 min) and DMTU (retention time 9.26 min) at 1 mg/l; injection volume, 5 μl. (A) Recorded in full-scan mode. (B) Recorded in selected-ion mode, according to Table I.

in Fig. 5A. With the GC–MS system operating in full-scan mode, the sensitivity is not high and the detection of some of the thiouracil analogues is disturbed by interfering compounds present in chemicals and the urine sample. In selected-ion mode, screening four ions per compound (Table I), the analytes can be distinguished clearly (Fig. 5B). The sensitivity is comparable with that of the GC–NPD technique, while the specificity is much higher. For confirmation purposes the GC retention time of the analyte has to be within ± 5 s and the intensity

TABLE I

IONS SELECTED FOR SCREENING WITH GC–MS

Thyreostatic compound	Ions (*m/e*)
TU	82, 109, 111, 156
MTU	83, 123, 125, 170
DMTU	83, 137, 139, 184
PTU	153, 170, 183, 198

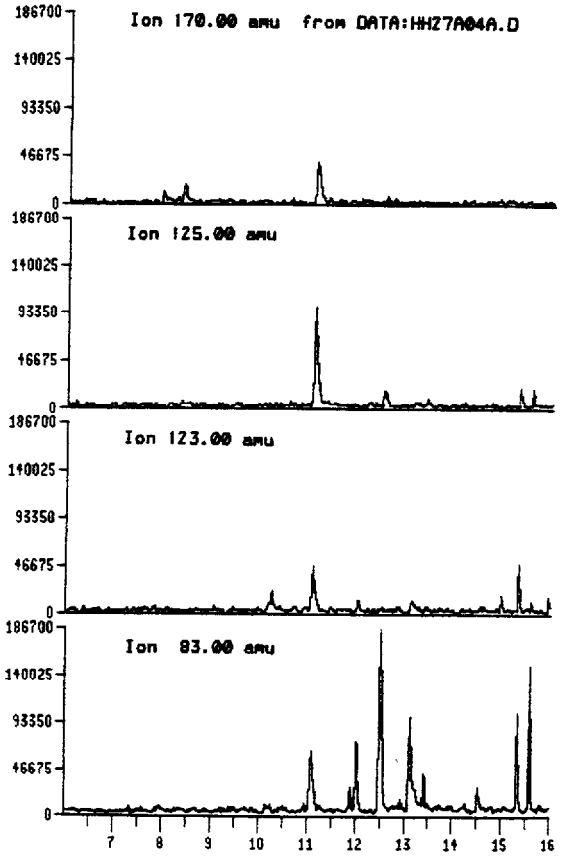

Fig. 6. Chromatograms (GC–MS) of the quality control sample (2 ml) containing MTU (retention time 11.1 min) (60 μg/l) after clean-up with the mercurated affinity column (injection volume, 5 μl), recorded for the four selected ions (Table I).

ratios of the four selected ions must be within ±10% when compared with the pure standard compounds, analysed in the same series [11,12].

On a later date, the MTU-containing quality control sample, with a concentration of 60 μg/l as determined by GC–NPD, was analysed in the selected-ion mode;

chromatograms of the four selected ions are shown in Fig. 6. According to the criteria mentioned above, MTU could be positively identified in this sample.

CONCLUSIONS

Using a selective mercurated affinity column combined with extractive alkylation as a derivatization procedure, the thiouracil analogues TU, MTU and PTU can be analysed by GC–NPD and GC–MS, in selected-ion mode, with excellent recoveries, permitting a detection limit in urine of 25 μg/l and providing a less difficult evaluation than the commonly applied HPTLC method. So far, the determination of TAP is problematic due to the low recovery from the affinity column and the low derivatization yield.

Future investigations will focus on the improvement of this technique with respect to the determination of TAP and the possibility of including another member of the thiouracil family, phenylthiouracil (PhTU).

Prior to the use of this GC approach in routine analysis, a validation according to the criteria mentioned in EC documents, concerning the quality aspects of screening and reference methods, has to be carried out [11,12].

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