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Determination of metabolites of pirimicarb in human urine by gas chromatography–mass spectrometry

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Abstract

The analytical method described permits the determination of 2-dimethylamino-5,6-dimethyl-4-hydroxypyrimidine (DDHP), 2-methylamino-5,6-dimethyl-4-hydroxypyrimidine (MDHP) and 2-amino-5,6-dimethyl-4-hydroxypyrimidine (ADHP) in human urine. These hydroxypyrimidines are metabolites of pirimicarb (2-dimethylamino-5,6-dimethylpyrimidin-4-yldimethylcarbamate) which is applied as insecticide. The analytes are extracted into a mixture of diethyl ether and acetonitrile. Pentafluorobenzyl bromide serves as derivatising reagent. The derivatives are analysed using capillary gas chromatography with mass selective detection. 2-Amino-4-hydroxy-6-methylpyrimidine and 4-hydroxy-6-trifluoromethylpyrimidine are used as internal standards. The detection limits are 0.5 μg/l (DDHP), 1 μg/l (MDHP) and 4 μg/l (ADHP), respectively. The method was used for analysing seven urine samples collected from workers who had applied pirimicarb. The three metabolites were found in every sample in concentrations up to 60 μg/l.

Keywords: Derivatization, GC; Pirimicarb; Hydroxypyrimidine

1. Introduction

Pirimicarb (2-dimethylamino-5,6-dimethylpyrimidin-4-yldimethylcarbamate) is an *N*,*N*-dimethylcarbamate. It was introduced in 1969 as selective insecticide and is applied against aphids in agriculture and fruit growing [1]. Inhibition of acetylcholinesterase is the basis for its insecticidal effect but causes toxicity for mammals including man as well [2,3]. The oral LD_{50} for rats is about 150 mg/kg body weight. It is classified as moderately hazardous and the acceptable daily intake for humans has been set to 0.02 mg/kg by the World Health Organisation (WHO) [4].

During application of pirimicarb workers are exposed to the insecticide to a certain extend even if they use protective equipment. Pirimicarb is sold as water soluble powder and in cans for fumigation. Due to its considerable vapour pressure [5] pirimicarb can be absorbed by inhalation especially at higher temperatures [6]. Threshold limit values for workplaces have not been established by the Deutsche Forschungsgemeinschaft (DFG) at present. Exposure assessment by means of air monitoring neglects the amounts of carbamates that are dermally absorbed [7]. Determination of actual exposure of an individual can be provided by conducting biological

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monitoring [8]. But according to our knowledge reliable procedures for biomonitoring of occupational exposure to pirimicarb have not been published yet.

Pirimicarb is metabolised rapidly in mammals. Experiments using rats and dogs have yielded similar results. Hydrolysis of the carbamate moiety and demethylation are the most important metabolic reactions. The major urinary metabolites are 2-dimethylamino-5,6-dimethyl-4-hydroxypyrimidine (DDHP), 2-methylamino-5,6-dimethyl-4-hydroxypyrimidine (MDHP) and 2-amino-5,6-dimethyl-4-hydroxypyrimidine (ADHP). Each of these metabolites accounts for 10 to 41% of the applied dose of pirimicarb [6]. They are depicted in Fig. 1. It is

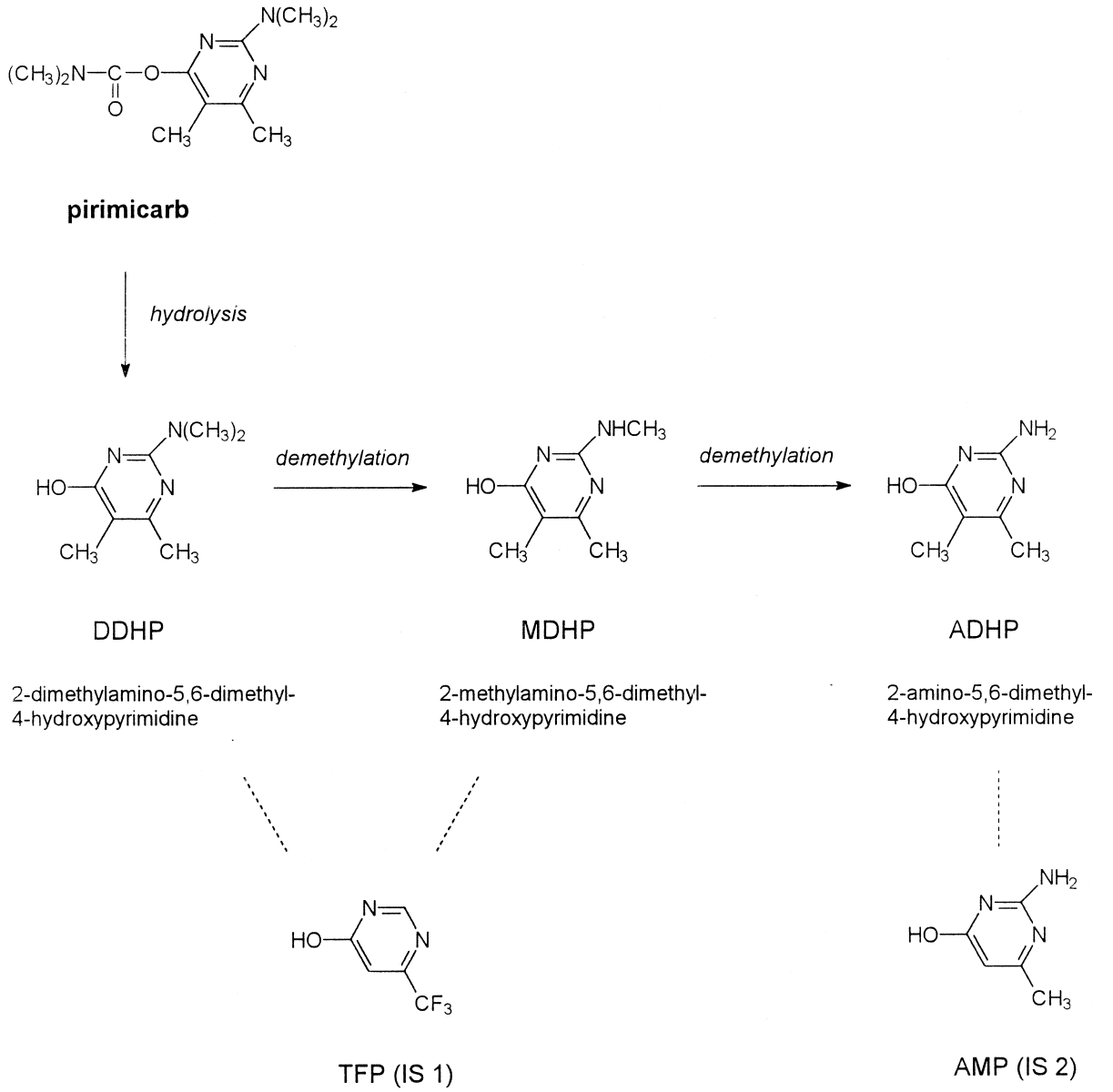

Fig. 1. Major urinary metabolites of pirimicarb in mammals and the internal standards used in the analysis (TFP: 4-hydroxy-6-trifluoromethylpyrimidine, I.S.: internal standard, AMP: 2-amino-4-hydroxy-6-methylpyrimidine).

worth mentioning that they are excreted unconjugated in urine. The only human study quantifying metabolites of pirimicarb in human urine was a pilot study published by Verberk et al. [9] and Sjardin et al. [10]. They found DDHP and MDHP in urine samples of three occupationally exposed workers in the low μg/l range using gas chromatography–mass spectrometry (GC–MS). The metabolite ADHP was not included in this analysis.

So the aim of our study was to develop a reliable analytical procedure for the simultaneous determination of the metabolites DDHP, MDHP and ADHP in human urine. The method and its reliability criteria are described and discussed in detail. It was checked by analysing seven urine samples collected from workers after applying pirimicarb and 10 urine specimens from persons without occupational exposure to pirimicarb.

2. Experimental

2.1. Chemicals

DDHP (certified assay: 99.8%), MDHP (95%) and ADHP (97.6%) were supplied by Promochem (Wesel, Germany). 2-Amino-4-hydroxy-6-methylpyrimidine (98%, AMP), 4-hydroxy-6-trifluoromethylpyrimidine (99%, TFP) and 2,3,4,5,6-pentafluorobenzyl bromide (99%, PFBBr) were purchased from Aldrich (Steinheim, Germany). Other chemicals and solvents used were of analytical grade and obtained from Merck (Darmstadt, Germany). Water was purified with a Milli-Q purification system (Millipore, Eschborn, Germany).

2.2. Solutions

Stock solutions of the internal standards (I.S.) TFP and AMP were prepared by dissolving 25 mg in 50 ml methanol each in separated flasks (concentration: 0.5 g/l). A 1-ml volume of each stock solution was pipetted into one 100-ml glass volumetric flask and diluted to the mark with water yielding the I.S. solution. This contained both internal standards at a concentration of 5 mg/l each and was used in sample preparation. The solution of PFBBr used for derivatisation was prepared by mixing 3 ml of PFBBr with 6 ml acetonitrile resulting in PFBBr–acetonitrile (1:2, v/v). This solution can be stored at least for six weeks at 5°C.

2.3. Sample preparation

The urine samples were collected in polypropylene bottles and stored at −18°C until sample preparation was carried out. After thawing and mixing 5 ml of urine were added to 4 g K_2HPO_4 in a vial with a screw top and spiked with 100 μl of I.S. solution. The sample was extracted with 5 ml of diethyl ether–acetonitrile (1:1, v/v) by mixing for 5 min followed by centrifugation at 1500 *g* for 5 min. The extraction step was repeated with further 5 ml of diethyl ether–acetonitrile (1:1, v/v) and the combined organic phases were evaporated to dryness at 40°C using a gentle stream of nitrogen. A 1-ml volume of acetonitrile was added and evaporated again in order to remove residual water. The analytes were derivatised in 1.5 ml acetonitrile by using 100 μl of PFBBr–acetonitrile (1:2, v/v) and 10 mg potassium carbonate in an oven at 80°C for 2 h. After cooling to ambient temperature 4 ml water were added and the sample was extracted with 1 ml heptane (mixing and centrifugation for 5 min each). The extraction was repeated with 1 ml heptane. The combined heptane phases were concentrated under a gentle stream of nitrogen to a final volume of 200 μl and analysed by GC–MS. The sample preparation is summarised in Fig. 2.

2.4. Gas chromatography–mass spectrometry

GC–MS was performed on a gas chromatograph HP 5890 series II connected to a HP 5989 A mass spectrometer (Hewlett-Packard, Waldbronn, Germany). A DB WAX capillary column (100% polyethylene glycol, 60 m×0.32 mm I.D., 0.25 μm from J&W Scientific, Folsom, CA, USA) was used. The conditions were as follows: carrier gas: helium 5.0, inlet pressure: 103 kPa, splitless injection of 1 μl. Temperatures: injector: 260°C, column: 100°C, raised at a rate of 6°C/min to 240°C, isothermal for 25 min. Interface: 280°C, ion source: 200°C, quadrupole: 100°C. Ionisation by electron impact (70 eV), electron multiplier voltage 2300 V. Mode: multiple ion detection.

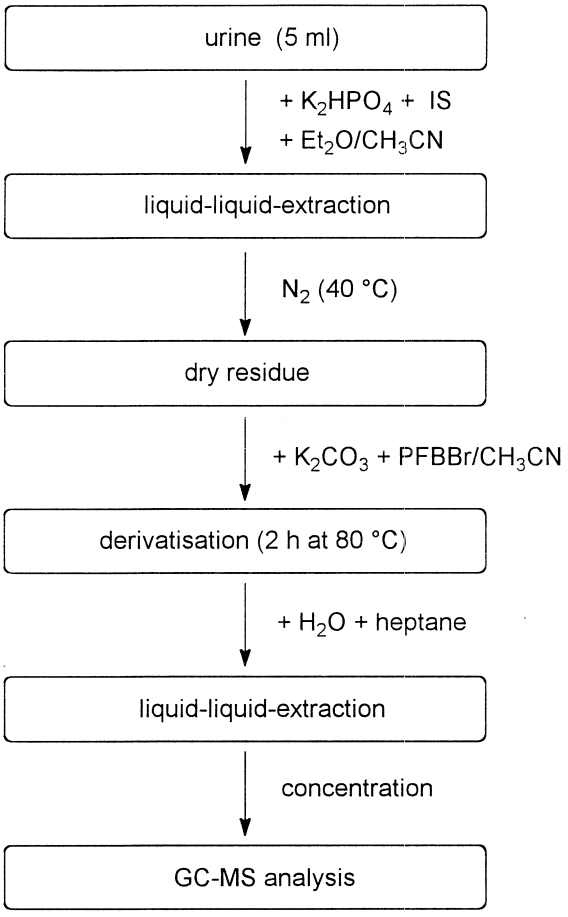

Fig. 2. Sample preparation.

The registered masses of the derivatives and their retention times are summarised in Table 1. The molecular ions were used for quantification.

Table 1
Retention times and monitored masses (I.S.: internal standard, PFB: pentafluorobenzyl)

PFB-derivatives of the analytes	Retention time (min)	Monitored masses (*m*/*z*)	Quantifier (*m*/*z*)
DDHP	21.0	347, 332, 299, 166	347
TFP (I.S. 1)	23.5	344, 325, 161	344
MDHP	24.0	333, 152, 123	333
AMP (I.S. 2)	25.8	305, 285, 161	305
ADHP	26.2	319, 161, 138	319

2.5. Calibration process and quality control

Three separate starting solutions were made by dissolving 25 mg of ADHP, MDHP and DDHP in 50 ml methanol, respectively. A 500-μl volume of each starting solution was transferred into one 50-ml glass volumetric flask and diluted to the mark with water yielding a stock solution that contains the three metabolites at a concentration of 5 mg/l each. Five calibration standards with concentrations ranging from 2 to 100 μg/l were prepared from this stock solution by diluting with pooled urine. This pooled urine had been collected from people without known exposure to pirimicarb. It was stored at −18°C and filtered once before use. Calibration standards were stable for more than six months at −18°C. They were processed together with an unspiked pooled urine as described. The ratio of the peak areas of an analyte and an I.S. was plotted against the concentration resulting in linear calibration curves. DDHP and MDHP were quantified with respect to the internal standard TFP whereas ADHP was analysed using the second internal standard AMP. The correlation coefficients were higher than 0.999.

Because no quality control material was commercially available it had to be prepared in the laboratory. We used two pools of urine after spiking with a stock solution containing ADHP, MDHP and DDHP in equal amounts. Pool 1 contained the three metabolites at a concentration of 15 μg/l each. The concentrations in pool 2 were 60 μg/l, respectively. The pools were divided into aliquots and stored at −18°C. For quality assurance two control samples of different concentrations were included in each analytical series.

2.6. Study subjects

In the present study we investigated seven urine samples of workers employed in agriculture and fruit plantation. They had applied the insecticide pirimor (pirimicarb) for 3.75 to 8.25 h. Urine was collected for 24 h after the end of exposure and stored at −18°C until it was processed. Creatinine content ranged from 0.8 to 2.3 g/l. Moreover five spot urine samples and five 24-h urine specimens from 10 persons without occupational exposure to pirimicarb were analysed for control purposes.

3. Results and discussion

According to our knowledge the method described is the first one that allows the determination of DDHP, MDHP and ADHP in human urine in one analytical run. The only method published so far [10] considered only the metabolites MDHP and DDHP and had not been checked with respect to accuracy and reproducibility.

3.1. Extraction of the urine and derivatisation

Because of the polarity of the metabolites a highly polar solvent has to be used for extracting the urine. Dichloromethane, ethyl acetate and pure diethyl ether were found to be unsuitable but a mixture of acetonitrile and diethyl ether resulted in a high yield of extraction. Because acetonitrile is miscible with water it is necessary to saturate the urine with a soluble salt in order to separate the organic phase. Using dipotassiumphosphate results in a pH of 9 so that analytes and I.S. are uncharged and can be extracted into diethyl ether–acetonitrile. Preliminary experiments revealed that pH values in the range from 6 to 10 are suitable. Some water is coextracted into the organic phase because of its high polarity. Therefore the solution is evaporated completely in order to make sure that the derivatisation is not influenced by residual water.

PFBBr has been used as derivatising reagent for phenols [11,12], chlorophenols [13] and isopropoxyphenol [14]. Sjardin et al. [10] applied PFBBr for derivatising DDHP and MDHP for the first time. Reactions of DDHP, MDHP, ADHP, TFP and AMP with PFBBr within our procedure result in monoderivatised products. The derivatives are supposed to be ethers because the oxygen is more nucleophilic than the aromatic amino moiety under the alkaline conditions described. We did not observe further derivatisation products of the analytes in the chromatograms. However, on the basis of our experimental results (GC–MS) we cannot exclude completely that a reaction takes place between PFBBr and the amino moiety of for example ADHP and AMP.

3.2. Internal standards

We used two internal standards which are commercially available. DDHP and MDHP are determined by relating their peak areas to internal standard 1 (TFP). ADHP is quantified using the second internal standard AMP. This procedure improves the precision of the analytical method because the metabolite ADHP and the internal standard AMP are very similar. Both contain a primary (and polar) amino group which might affect yields of extraction and derivatisation. TFP proved to be a suitable internal standard for DDHP and MDHP because these three molecules do not contain a primary amino moiety. Metabolites of pirimicarb and I.S. are depicted in Fig. 1.

Please note that AMP is a possible metabolite of the organophosphates pirimiphos-methyl and pirimiphos-ethyl which are applied as insecticides [15]. Hydrolysis of the ester moiety and subsequent two-fold dealkylation of the diethylamino moiety can result in AMP (Fig. 3). Therefore it might be necessary to check urine specimens with respect to their content of AMP (without adding I.S. solution) if exposure to the above mentioned organophosphates has taken place. This was a further reason to add two I.S., because in the case of a coexposure to

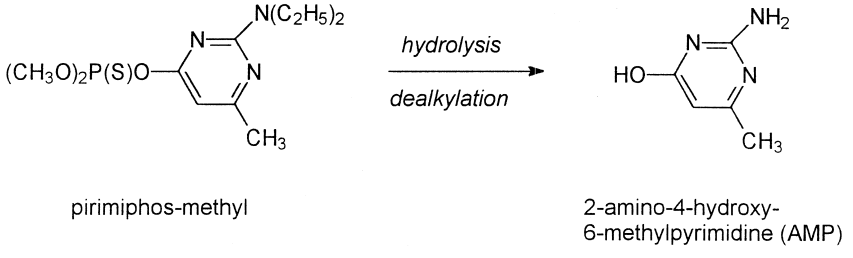

Fig. 3. Pirimiphos-methyl as possible source of AMP.

pirimicarb and pirimiphos the metabolites of pirimicarb can be determined by using only the internal standard TFP. However, excretion of AMP has never been reported so far after exposure of humans to pirimiphos-methyl or its ethyl analogue.

3.3. GC–MS analysis

A capillary column with a polyethylene glycol film (DB WAX, 60 m) allowed us to separate the analytes from one another and the interfering background. It is noteworthy that a HP Innowax-column (60 m×0.32 mm I.D., film thickness 0.25 μm) which has a very similar film proved to be unsuitable for GC. Using this column for analysing standard solutions (50 mg/l) the derivatives of MDHP, ADHP and AMP could not be detected. The reason for that phenomenon remains unclear but it might be an effect of the primary or secondary amino moiety. Another column with a 35% diphenyl–65% dimethylpolysiloxane phase (HP 35, 60 m×0.25 mm I.D., film thickness 0.25 μm) proved to be suitable for GC analysis.

Pentafluorobenzyl (PFB) derivatives of the metabolites and I.S. are registered by at least three masses including the molecular ions (Table 1). Two fragment ions of each analyte are still detectable at the limit of detection. This results in a highly specific analysis identifying the analytes both by their retention times and the mass ratios.

As an example the mass spectrum of MDHP-PFB is shown in Fig. 4.

3.4. Reliability of the method

3.4.1. Precision

The within-series imprecision was determined by analysing pooled urine six times after spiking with the three metabolites at a concentration of 15 μg/l each. Moreover within-series imprecision was determined with eight individual urine samples from different people without previous exposure to pirimicarb. These specimens were checked to contain

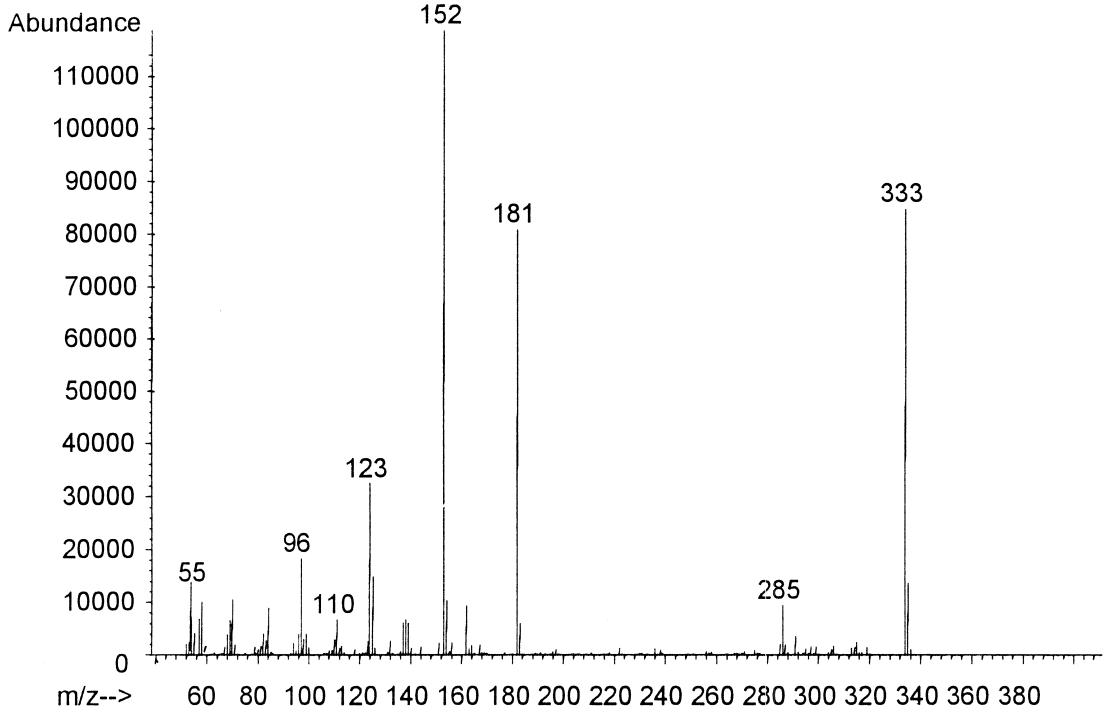

Fig. 4. Mass spectrum of derivatised 2-methylamino-5,6-dimethyl-4-hydroxypyrimidine (MDHP-PFB) m/z 333: M^+ (molecular ion), m/z 181: PFB^+, m/z 152: M^+-PFB, m/z 123: M^+-PFB-NCH_3.

Table 2
Imprecision of the method as relative standard deviations (spiked pool urine; *=spiked individual urine specimens of unexposed persons)

Metabolite	Within-series imprecision (%) at,		Between-day imprecision (%) at,	
	15 μg/l (n=6)	30 μg/l (n=8)*	15 μg/l (n=8)	60 μg/l (n=8)
ADHP	7.6	9.4	16.2	10.5
MDHP	6.0	9.0	14.4	11.8
DDHP	7.2	9.6	9.4	10.6

no metabolites above the detection limit and were spiked afterwards resulting in a content of 30 μg/l of each metabolite. The creatinine content ranged from 0.2 to 1.7 g/l. The relative standard deviations (RSDs) of the within-series imprecision were below 10% (Table 2).

Between-day imprecision was determined by analysing both quality control samples at eight different days. Results are given in Table 2. The RSDs were below 15% except for ADHP at a concentration of 15 μg/l. This exception can be explained by the fact that the analysed concentration was only about four-times the detection limit of ADHP.

3.4.2. Accuracy

In order to check the accuracy recovery experiments were carried out at concentrations of 15, 30 and 60 μg/l. Recovery was calculated after analysing spiked urine samples six or eight times as described and comparing the results with the spiked amounts. The unspiked urine was checked to contain no metabolites above the detection limit. Recoveries of DDHP, MDHP and ADHP ranged from 81 to 119% (Table 3).

Losses during the whole analytical procedure could not be determined because the PFB derivatives of the pyrimidines have not been available. Efficiency of the extraction of the urine with subsequent evaporation of the solvent was examined as follows. A solution of DDHP, MDHP and ADHP in acetonitrile was derivatised directly. After extraction in heptane and concentrating to 200 μl analysis was carried out by GC–MS. Results were compared with those obtained from a processed urine sample which contained the same amounts of the metabolites as the solution in acetonitrile. In order to compensate differences in final volumes hexachlorobenzene was used as internal standard in this experiment. Calculated losses due to extracting the urine and evaporation to dryness were below 10% for DDHP, MDHP and ADHP at a concentration of 50 μg/l urine each.

The influence of the urinary matrix on the analysis was examined by comparing calibration graphs for urine and water. Aqueous and urinary calibration standards were prepared in the same manner described above. The aqueous calibration graphs proved to be also linear. Their coefficients of correlation were at least 0.997. The slope of the aqueous calibration graphs of DDHP, MDHP and ADHP in relation to the urinary ones were 85%, 78% and 91%, respectively. This indicates that it is advisable to perform the calibration procedure using standards in urine.

3.4.3. Detection limit

The limits of detection defined as signal-to-noise ratio of three concerning the molecular ions are 0.5 μg/l (DDHP), 1 μg/l (MDHP) and 4 μg/l (ADHP). At these concentrations two ions of each analyte are

Table 3
Accuracy of the method as relative recovery (spiked pool urine; *=spiked individual urine specimens of unexposed persons)

Metabolite	Within-series accuracy (%) at,		Between-day accuracy (%) at,	
	15 μg/l (n=6)	30 μg/l (n=8)*	15 μg/l (n=8)	60 μg/l (n=8)
ADHP	81	88	93	91
MDHP	103	94	111	106
DDHP	113	104	119	100

detected (DDHP-PFB: *m/z* 347, 166; MDHP-PFB: 333, 152; ADHP-PFB: 319, 138). No blank values caused by the reagents were detected.

3.5. Examination of exposed persons

Fig. 5 shows the chromatogram of a processed urine sample which had been collected from a worker after application of pirimicarb. The urine contained 1 μg/l DDHP, 43 μg/l MDHP and 10 μg/l ADHP.

The results of biomonitoring are summarised in Table 4. Every urine sample derived from occupational exposed workers contained all three metabolites. The demethylated metabolites MDHP and ADHP were excreted in significant higher amounts than DDHP. The median values of the concentrations in μg/l are higher by a factor of about 30 and seven, respectively. It is worth mentioning that no worker complained about acute adverse health effects.

Table 4
Concentrations of metabolites in urine after occupational exposure ($n=7$)

	DDHP (μg/l)	MDHP (μg/l)	ADHP (μg/l)
Range	0.9–2.2	26.1–60.0	4.2–17.3
Median	1.5	40.0	10.9

Furthermore urine samples from workers after exposure were analysed without using I.S. They did not contain TFP or AMP.

The only previous study in which urinary metabolites after exposure to pirimicarb were found was

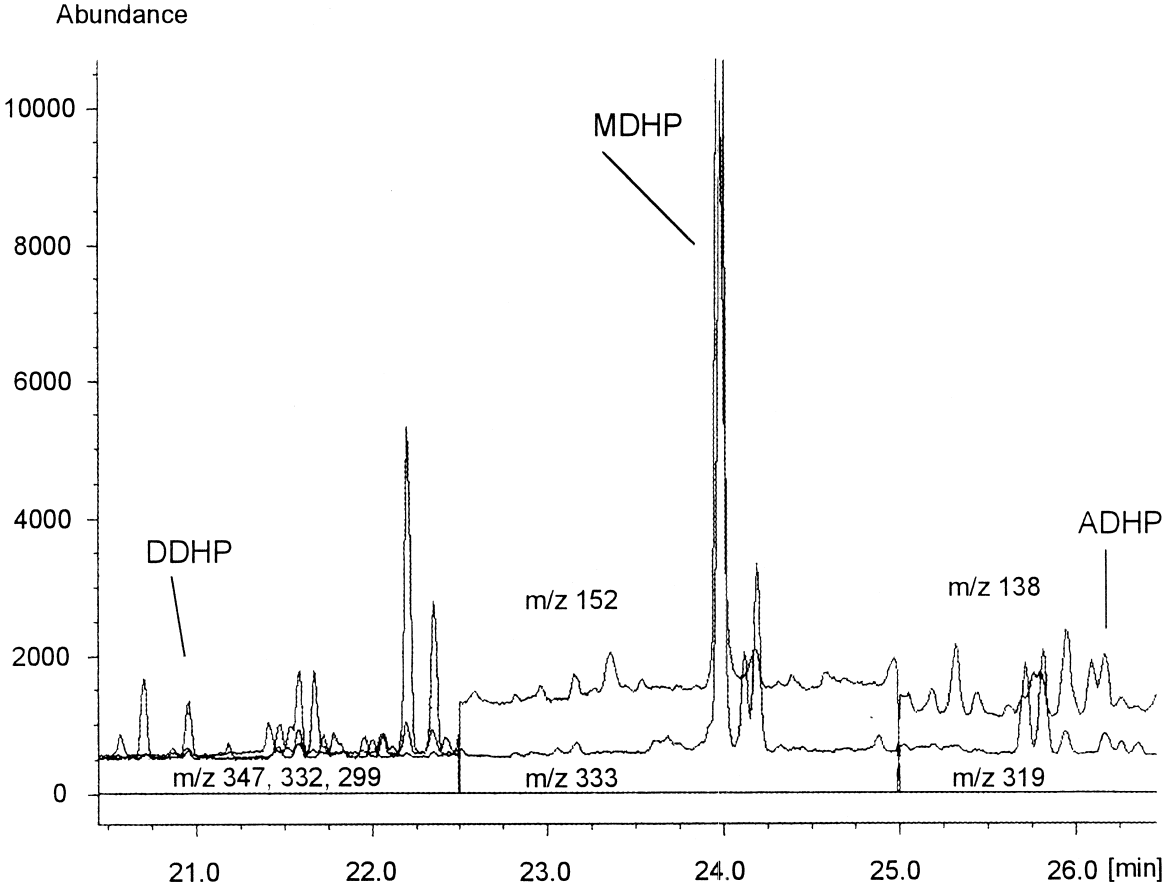

Fig. 5. Chromatogram of a processed urine sample of a worker after application of pirimicarb (DDHP 1 μg/l, MDHP 43 μg/l, ADHP 10 μg/l, *m/z* of internal standards not shown).

conducted in the Netherlands by Sjardin et al. Only the two metabolites DDHP and MDHP were investigated in the urine of three workers using a method which had not been validated completely [9,10]. Urinary excretion ranged up to about 100 μg/l. MDHP was prevailing by a factor of 10 to 50. Our results agree very well with this pilot study. In another study neither DDHP nor MDHP were detected in urine samples of greenhouse workers [7]. Up to now no further human studies have been published according to our knowledge.

Concerning the controls we were not able to detect metabolites in any urine sample without previous exposure during application. Therefore the metabolites investigated represent diagnostically sensitive and specific parameters for the estimation of an individual uptake of pirimicarb during application. A chromatogram of a processed urine sample derived from a person without contact with pirimicarb is depicted in Fig. 6.

4. Conclusion

We have developed a reliable, sensitive and selective analytical method for the simultaneous determination of three pirimicarb metabolites in human urine. The results of our study indicate that the metabolites DDHP, MDHP and ADHP are suitable parameters for biological monitoring of occupational exposure to pirimicarb.

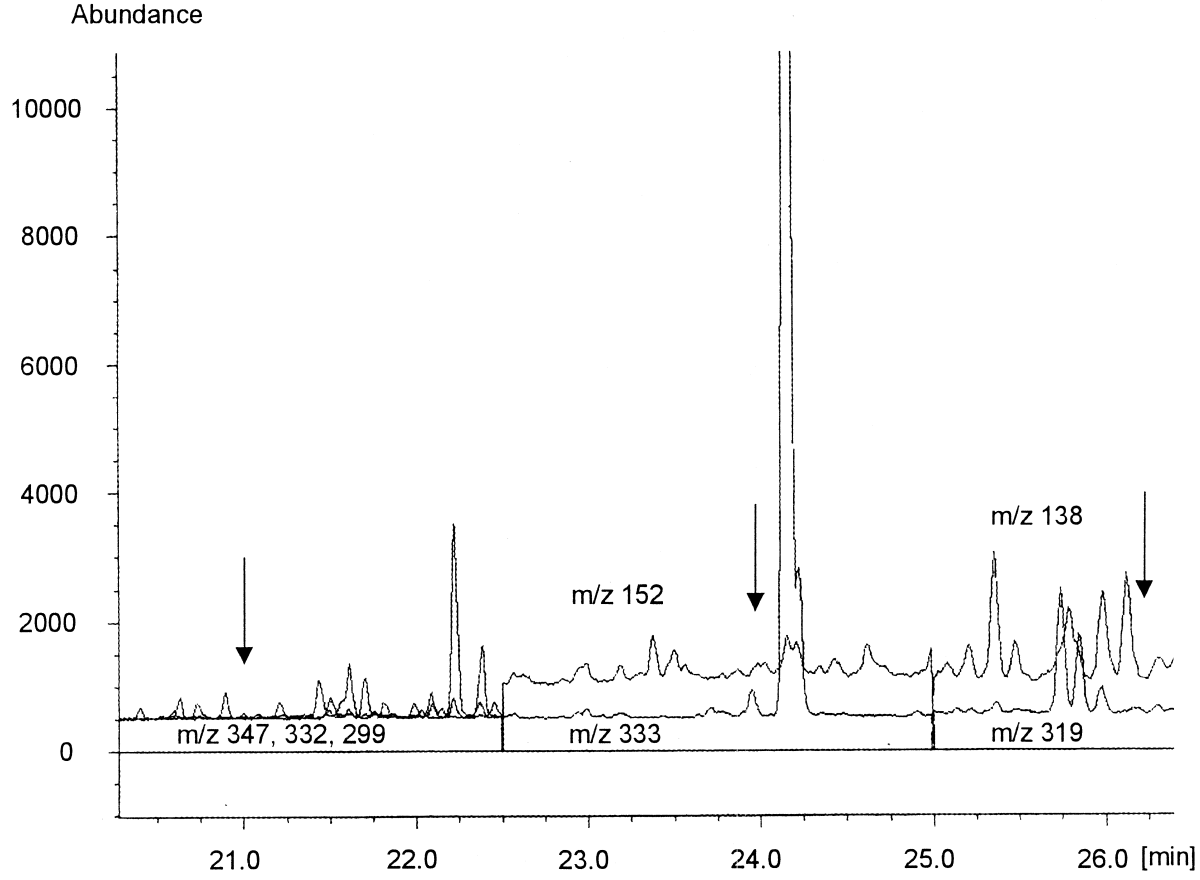

Fig. 6. Chromatogram of a processed urine sample of a person without exposure (m/z of internal standards not shown, retention times of the metabolites are marked by arrows).

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