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Gas chromatographic method with mass-selective detection for the determination of 2-isopropoxyphenol in human urine

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Abstract

Human metabolism of the insecticide propoxur yields 2-isopropoxyphenol (IPP) which is excreted conjugated in urine. In this publication a sensitive and selective analytical method is described which permits the determination of IPP as a suitable parameter for biomonitoring. The clean-up of the hydrolysed urine samples consisted of steam distillation and solid-phase extraction using a reversed-phase column. IPP and the internal standard 2-ethoxyphenol were converted to their pentafluorobenzyl ethers. Excess of the derivatisation reagent was removed using deactivated silica gel. Separation and quantitative analysis was carried out by capillary gas chromatography and mass selective detection. Coefficients of variation were below 5% for concentrations from 6 to 300 μg/l. The detection limit was 0.5 μg/l. The method was checked by analysing six urine samples from pest controllers after indoor application of propoxur. The IPP concentrations ranged from 45 to 306 μg/g creatinine. IPP was not detected in urine specimens from 10 non-exposed persons. The sensitivity of the developed method permits the detection of latent exposure to propoxur.

Keywords: 2-Isopropoxyphenol; Propoxur

1. Introduction

Propoxur (2-isopropoxyphenyl methylcarbamate) was introduced by Bayer in 1959 as a non-systemic insecticide. It has a rapid knock-down effect on insects and is applied against pests in agriculture and households [1]. Its acute toxicity is due to inhibition of acetylcholinesterase both for insects and for mammals. It is classified as moderately hazardous by the World Health Organization (WHO). The oral LD_{50} for rats is about 90 mg/kg [2] and the acceptable daily intake for humans has been set at 0.02 mg/kg by the WHO [3].

Estimation of health risks requires an assessment of exposure. Biological monitoring provides the determination of the actual absorbed amount of a compound in humans. This is especially important in the case of pesticides like propoxur because it is readily absorbed through the skin [4]. Dermal exposure is difficult to estimate by means of ambient monitoring [5]. Furthermore, the determination of a compound or a suitable metabolite in body fluids presents the possibility of detecting unknown routes of non-occupational exposure.

Propoxur is metabolised rapidly in mammals [6,7]. The most important metabolic pathway in humans is

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shown in Fig. 1. Hydrolysis of the carbamate linkage yields 2-isopropoxyphenol (IPP) which is excreted as glucuronide in urine [8]. Eighty-eight percent of orally ingested propoxur (0.4 to 7 mg) could be found as conjugated IPP in a study of Meuling et al. [9] with 17 volunteers. A linear relationship between propoxur dose and total excretion of IPP in urine after oral as well as after dermal application was also confirmed. This indicates the suitability of IPP as a parameter for biomonitoring of propoxur exposure.

Analytical methods for the determination of IPP in human urine published so far do not provide sufficient sensitivity [10–12] or require tandem mass spectrometry [13,14] which is lacking in many laboratories. Our aim was to obtain a procedure with a limit of detection below 1 μg/l in order to be able to detect any exposure which exceeds the 95th percentile of the general population. This limit was determined in the USA by Hill et al. to be 1.6 μg/g creatinine [15].

We developed a reliable and highly sensitive method for the determination of IPP in human urine samples using capillary gas chromatography and mass-selective detection. The method was checked by analysing six urine samples from workers after occupational application of propoxur and from 10 persons without occupational exposure to pesticides.

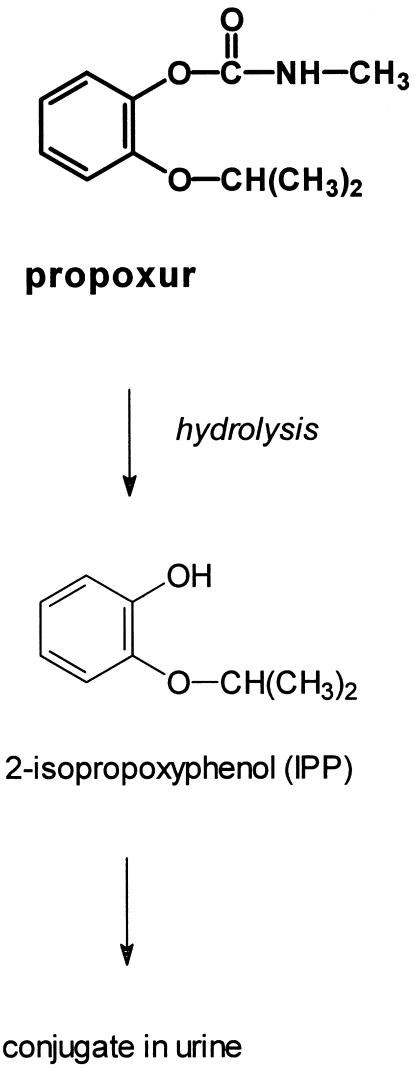

Fig. 1. Main metabolic pathway of propoxur in humans.

2. Experimental

2.1. Chemicals and materials

2-Isopropoxyphenol (certified assay: 98.4%) was supplied by Dr. Ehrenstorfer (Augsburg, Germany). 2-Ethoxyphenol (98%) and 2,3,4,5,6-pentafluorobenzyl bromide (PFBBr, 99%) were obtained from Aldrich (Steinheim, Germany). Acetone, acetonitrile, cyclohexane, heptane, hydrochloric acid (37%), methanol, toluene, potassium carbonate, and anhydrous sodium sulphate were supplied by Merck (Darmstadt, Germany). All chemicals and solvents used were of analytical grade. Water was purified with a Milli Q purification system (Millipore, Eschborn, Germany). Steam distillation was carried out using the Vapodest 20® (Gerhardt, Bonn, Germany).

The stock solution of the internal standard (IS) 2-ethoxyphenol was prepared by dissolving 50 mg in 50 ml methanol (1 g/l). This stock solution was diluted with water to a concentration of 10 mg/l. The resulting IS solution was used for spiking urine samples.

Polypropylene columns (empty reservoirs, 3 and 50 ml) for solid-phase extraction (SPE) and 20 μm polyethylene frits were purchased from ICT (Bad Homburg, Germany). Reversed-phase octadecyl-modified silica (C_{18}, endcapped, average particle size 40 μm, pore diameter 60 Å) and silica gel (average particle size 40 μm, pore diameter 60 Å) bulk

packings were obtained from Baker (Deventer, The Netherlands).

Each column (3 ml) for reversed-phase SPE was filled with 0.5 g C_{18} adsorbent surrounded by two frits. Conditioning was carried out with 5 ml acetonitrile and 5 ml water. The sorbent was kept wet until the sample was added. Silica gel was activated at 150°C for 24 h. Afterwards it was deactivated by adding 20% water (w/w) and shaking for 2 h. Columns (3 ml) for normal-phase SPE were filled with one frit, 400 mg silica gel and 300 mg anhydrous sodium sulphate. Conditioning was performed with 5 ml cyclohexane.

2.2. Gas chromatography–mass spectrometry (GC–MS)

GC–MS was performed on a gas chromatograph HP 5890 Series II equipped with a split/splitless injector, an autosampler HP 7673, and a mass-selective detector HP 5972 (Hewlett-Packard, Waldbronn, Germany). One microlitre of each sample was injected in the splitless mode with a purge off time of 1 min. The operating temperature of the injection port was 260°C. A 35% diphenyl–65% dimethylpolysiloxane capillary column (HP 35), 60 m×0.25 mm I. D., film thickness 0.25 μm (Hewlett-Packard) was used. The initial column temperature of 120°C was held for 1 min, then raised at a rate of 5°C/min to 160°C, held at this temperature for 32 min, and raised at a rate of 30°C/min to 250°C, remaining at this temperature for 14 min. The carrier gas was helium 5.0 at a constant flow of 0.6 ml/min. The temperature of the transfer line was maintained at 300°C. The mass-selective detector was operated at an electron energy of 70 eV and an electron multiplier voltage of 2300 V.

Multiple ion detection was used. The masses m/z registered were 332, 290, and 161 for the pentafluorobenzylether of IPP (IPP-PFB) and 318, 161, and 137 for the pentafluorobenzylether of IS (IS-PFB). The retention times were 37.9 and 38.6 min, respectively. The molecular ions (m/z 332 and 318) were used for quantification.

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 was pipetted into a vial with a screw top and spiked with 100 μl of IS solution. Acidic hydrolysis of the conjugated IPP was performed by adding 2 ml hydrochloric acid (18.5%) and heating for 90 min at 80°C in an oven. The sample was cooled to ambient temperature and diluted with 10 ml water. Afterwards steam distillation was carried out. The distillate (approximately 50 ml) was passed through a preconditioned C_{18} column on a Vac Elut vacuum manifold at normal pressure using additional reservoirs (50 ml). The column was dried for 20 min under full vacuum (15 in. Hg) and eluted by 1.4 ml acetonitrile. Derivatisation was performed in sealed vials in an oven at 70°C for 2 h after adding approximately 10 mg potassium carbonate and 100 μl PFBBr in acetonitrile (1:2, v/v). After cooling to ambient temperature, 1 ml *n*-heptane and 2 ml water were added. The pentafluorobenzyl ethers were extracted by mixing for 5 min followed by centrifugation at 1500 *g* for 5 min. Heptane phase (750 μl) was transferred to a preconditioned silica gel column. The column was washed with 4 ml cyclohexane and eluted with 3 ml cyclohexane–acetone (1:1, v/v) into a vial which already contained approximately 100 mg anhydrous sodium sulphate. Toluene (200 μl) was added and the solution was concentrated to a volume of 1.5 ml using a gentle stream of nitrogen. The solution was then transferred into a smaller vial and concentrated to a final volume of 200 μl for subsequent analysis by GC–MS. The sample preparation is summarised in Fig. 2. It is possible to interrupt the procedure after the steam distillation or after eluting of the reversed-phase SPE. The distillate or the eluate should be stored at 4°C overnight.

2.4. Calibration and quality control

A starting solution of IPP was prepared by dissolving 50 mg IPP in 50 ml methanol (1 g/l). This solution (500 μl) was diluted to a final volume of 100 ml with water yielding a stock solution with a concentration of 5 mg/l. Seven calibration standards with concentrations ranging from 1 to 300 μg/l were prepared from this stock solution by diluting with pooled urine collected from people without known exposure to propoxur. The calibration standards were stable for more than 12 months at −18°C. Linear

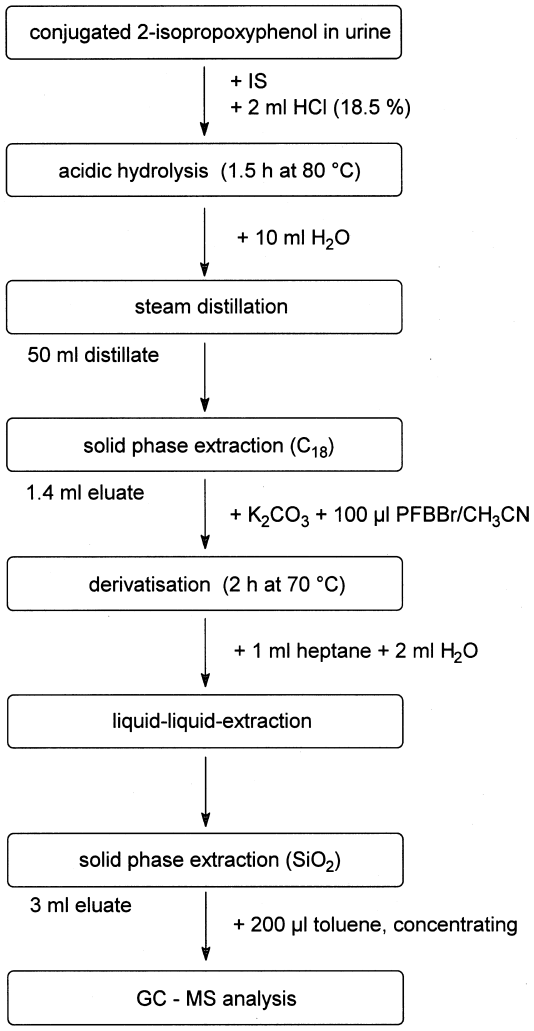

Fig. 2. Sample preparation.

calibration curves were obtained by plotting the ratio of the peak areas of IPP and IS as a function of the concentrations used. The correlation coefficients were >0.998.

For quality assurance two control samples were included in each analytical series. Because no quality control material was commercially available we used spiked pooled urine at a concentration of 12.3 µg/l and a 24-h urine specimen collected from a worker after propoxur application in agriculture (concentration 226 µg/l). Both urine specimens were divided into aliquots and stored at −18°C.

2.5. Study subjects

In the present study we investigated six urine specimens of workers employed in indoor pest control. They had applied propoxur for 0.25–0.75 h wearing simple protective clothing. Urine was collected for 24 h after the end of exposure and stored at −18°C until it was processed. Moreover, 10 spot urine samples of persons without occupational exposure to pesticides were analysed for control purposes.

3. Results and discussion

Cholinesterase inhibition in mammals after exposure to propoxur is reversible and only of short duration [16]. Therefore, the activity of acetylcholine esterase is not a suitable parameter for biological monitoring after occupational exposure to propoxur. In contrast, the determination of IPP in urine can be applied even after low-dose exposure. This requires a very sensitive analytical method.

PFBBr was chosen as the agent for derivatisation for the following reasons. Preliminary experiments revealed that IPP-PFB could be detected more sensitively by MS than the reaction product of pentafluoropropionic anhydride. Diazomethane was not suitable at all. Moreover, PFBBr yields ethers which are very stable compounds. Our own experiments indicated that samples prepared for GC–MS could be stored for at least 5 days at ambient temperature. This might be necessary if it is not possible to analyse them immediately. The conditions of the derivatisation reaction have been optimised with respect to practicality and yield. Temperatures lower than 70°C demanded an increased reaction time. Using smaller amounts than 100 µl PFBBr–acetonitrile (1:2, v/v) resulted in low and varying yields.

Our procedure contains a very effective clean-up using steam distillation and two different solid-phase extractions. This enables us to separate the analyte from interfering matrix components and to enrich it by a factor of 25. The main reason for applying SPE with silica gel after derivatisation is to remove the excess PFBBr before injection into the GC–MS system. This purification step is advisable when

many samples have to be analysed in order to protect the capillary column and the detector.

3.1. GC–MS analysis

We were able to separate the derivatives of IPP and 2-ethoxyphenol (IS) from the analytical background interference using a 35% diphenyl–65% dimethylpolysiloxane capillary column. A 5% diphenyl–95% dimethylpolysiloxane phase is less suitable for separating the analytes from derivatised benzoic acid which is excreted in significant amounts by humans and is not removed by the sample clean-up. Moreover, hippuric acid in urine is hydrolysed during the sample preparation and converted to benzoic acid.

Analyte and IS are registered by three fragment ions each including the molecular ions. Two fragment ions of 2-isopropoxyphenyl pentafluorobenzylether (IPP-PFB) are still detectable at the limit of detection. This results in a highly specific analysis identifying the analyte both by its retention time and the mass ratio of the investigated ions. The mass spectrum of IPP-PFB is shown in Fig. 3 (m/z 332, molecular ion M^+; m/z 290, $M^+–C_3H_6$; m/z 181, PFB; m/z 161, PFB-HF). The ion at m/z 181 is derived from nearly every analyte derivatised by PFBBr. Because it lacks selectivity and causes background noise it was not monitored.

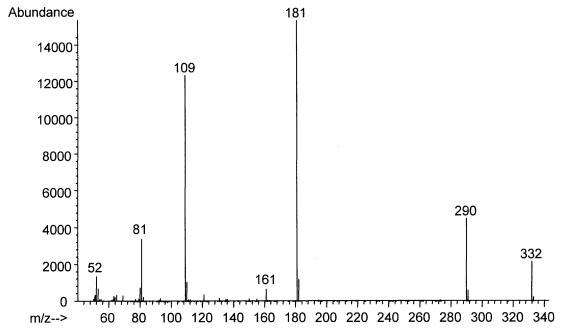

Fig. 3. Mass spectrum of 2-isopropoxyphenyl pentafluorobenzylether (IPP-PFB).

3.2. Reliability of the method

3.2.1. Precision

In order to determine the within-series imprecision pooled urine of people not exposed to propoxur was spiked with two different amounts of IPP. This resulted in two pools containing 6.2 and 300 μg/l, respectively. They were analysed eight times each. The relative standard deviations were calculated as 1.5% for the lower concentration and 4.4% for the higher concentration.

To provide even more realistic information about the precision of our method within-series imprecision was determined with individual urine samples from different people. Urine samples from six persons without previous exposure to propoxur were checked to contain no IPP above the detection limit. They were then spiked with IPP resulting in a concentration of 19.2 μg/l. The creatinine content ranged from 0.69 to 2.21 g/l. We obtained a relative standard deviation of 3.6%. This indicates that there is no relevant interference from different urine matrices to the precision of our method due to the very efficient clean-up procedure. The similarity of IS and IPP is assumed to be another reason for the good precision.

The between-day imprecision was determined by analysing both quality control samples on eight different days. The relative standard deviations were 4.6% for the spiked urine (12.3 μg/l) and 3.6% for the sample after propoxur exposure (226 μg/l).

3.2.2. Accuracy

Recovery experiments were carried out in order to check the accuracy. Pooled urine of persons without exposure to pesticides was spiked with IPP resulting in three subpools with different concentrations. Each subpool was analysed eight times. The relative recovery was 106, 95 and 105% at concentrations of 6.2, 12.3 and 300 μg/l, respectively. IPP could not be detected in the unspiked pooled urine.

Comparing calibration graphs for urine and water revealed negligible differences. Aqueous and urinary calibration standards were prepared in the same manner. The slope of the linear calibration graphs in water ($y = 0.002447x$) was on average only 2% higher than the slope of the urinary ones ($y = 0.002408x$). This indicates that aqueous calibration standards may be used in future.

In order to check for losses which occur during sample preparation a solution of IPP in acetonitrile

was derivatised. The ether was extracted in heptane and analysed by GC–MS. The result was compared with those obtained from a processed urine sample which contained the same amount of IPP. In order to compensate for differences in final volumes hexachlorobenzene was used as an additional internal standard. The calculated losses due to processing except for derivatisation and extraction in heptane were 14% at a concentration of 100 μg/l IPP. Considering the sophisticated sample preparation these losses can be described as small. They are completely compensated by the applied calibration procedure. The yield of the derivatisation reaction could not be determined as the pentafluorobenzyl ether of IPP was not available.

3.2.3. Detection limit

The limit of detection defined as three times the signal-to-noise ratio was 0.5 μg/l. At this concentration two ions are still detectable (*m/z* 332 and 290).

3.2.4. Sources of error

No blank reagent values were detected. In order to avoid contamination by the analyte during the steam distillation it is necessary to clean the Vapodest 20® by distilling purified water after each sample.

3.2.5. Hydrolysis

The hydrolysis of the conjugate was investigated with respect to the amount of hydrochloric acid used. A urine sample of a worker after propoxur application in agriculture (containing 185 μg/l conjugated IPP) was aliquoted and analysed after performing acidic hydrolysis with different amounts of hydrochloric acid (18.5%). From the results shown in Fig. 4 it was concluded that hydrolysis can be performed at 80°C for 1.5 h with 0.25–3 ml acid corresponding to concentrations ranging from 0.3 to 2.3 mol/l. Leenheers et al. used 0.43 mol/l hydrochloric acid for 1 h at 100°C [12]. They recovered 80% of an orally administered dose of propoxur as IPP, indicating that probably all of the conjugated IPP was liberated after hydrolysis.

3.3. Examination of exposed persons

In order to check the developed analytical method urine samples from pest controllers after application

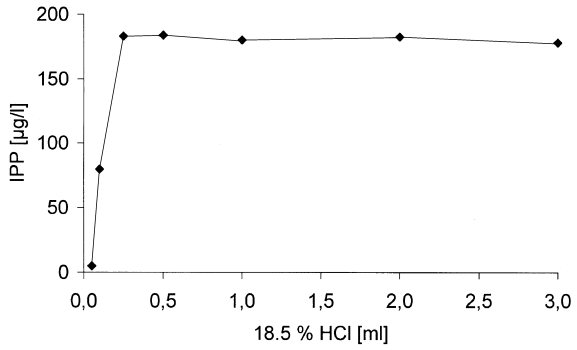

Fig. 4. Hydrolysis of 5 ml urine containing 185 μg/l conjugated IPP with various amounts of hydrochloric acid (18.5%).

of propoxur were examined. The results are shown in Table 1. A typical gas chromatogram of a urine sample from a worker after propoxur exposure is depicted in Fig. 5A. Brouwer et al. [5] monitored 16 workers engaged in harvesting in greenhouses where propoxur had been applied the previous day. IPP was excreted in amounts ranging from 10 to 1231 μg in 24 h. Lewalter and Korallus [17] found IPP in urine of seven plant workers exposed during manufacturing of propoxur in concentrations ranging from 200 to 2400 μg/g creatinine.

Although the workers in our study had used propoxur only during short periods of time wearing protective clothing they excreted IPP in considerable amounts (45–306 μg/g creatinine).

Ten urine specimens from persons without occupational exposure to propoxur did not contain IPP above the limit of detection (0.5 μg/l). This is in accordance with the results of Hill et al. who determined the 95th percentile of the general population of the USA as 1.6 μg/g creatinine [15]. Fig. 5B shows a typical chromatogram of a urine sample from a person not exposed.

Table 1
Concentrations of IPP in urine of workers after exposure

No.	IPP (μg/l)	IPP (μg/g)
1	37	45
2	169	95
3	170	91
4	82	195
5	129	159
6	171	306

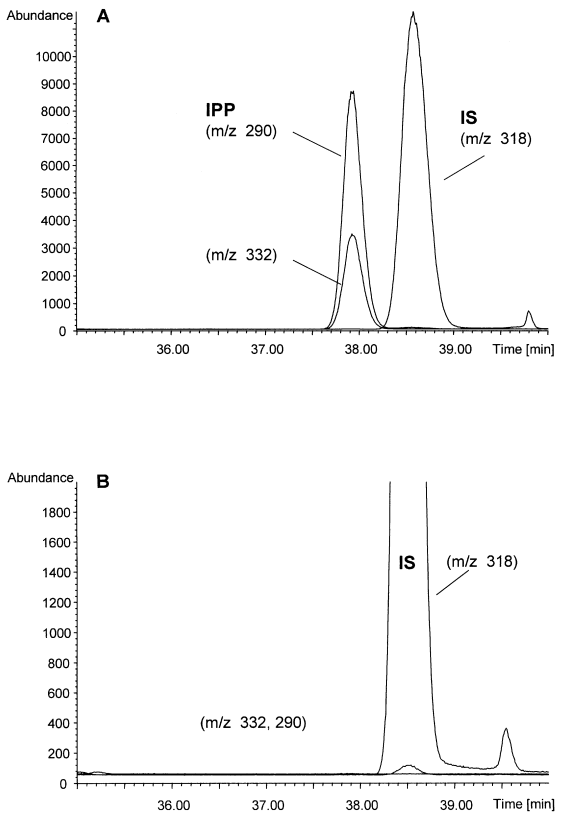

Fig. 5. (A) Processed urine sample of a worker after exposure to propoxur (IPP 171 μg/l) and (B) processed urine sample of a person without exposure to propoxur.

4. Conclusions

In summary, we were able to establish a reliable method for the determination of IPP in human urine with a detection limit of 0.5μg/l. This enables us to detect latent exposure to propoxur. The method was checked by analysing urine specimens of workers after propoxur exposure. To investigate possible exposure of the general population the sensitivity of the method must be increased. The efficiency of the clean-up procedure using steam distillation indicates that a detection limit of 0.1μg/l is possible by analysing 25 ml of urine.

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