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Determination of 2-isopropoxyphenol in urine by capillary gas chromatography and mass-selective detection

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ABSTRACT

An analytical method for the assessment of the exposure of workers to the pesticide propoxur through biological monitoring has been developed. This study was part of a survey of occupational exposure to pesticides used in greenhouses for the growing of ornamental plants. In order to assess the actual absorbed amount of propoxur in the body, an analytical method for its metabolite 2-isopropoxyphenol in urine was required. This led to the development of a gas chromatographic–mass spectrometric assay involving hydrolysis and solvent extraction. A mass-selective detector, operated in single-ion mode, provides a selective and sensitive quantification of 2-isopropoxyphenol with a detection limit of 6 μg/l. The method has been validated with respect to the hydrolysis of urine samples, analytical recovery of 2-isopropoxyphenol, stability of its conjugates, limit of detection, background and precision. The analytical recovery from spiked urine was over 95%. 2-Isopropoxyphenol was excreted in urine as a conjugate and was stable for at least seven months when stored at −20°C. It was not detected in urine from non-exposed persons. Between-day coefficients of variation were 20, 10, 7 and 4% for concentrations of 15, 29, 150 and 213 μg/l, respectively. Measured as 2-isopropoxyphenol, *ca.* 80% of an orally administered dose of propoxur was excreted in urine within 10 h.

INTRODUCTION

Approximately 30 000 workers in The Netherlands are involved in the culturing of ornamental plants in greenhouses. They are potentially exposed to a wide variety of pesticides during application and crop activities, which may represent a health hazard. This prompted the start of exposure studies in these greenhouses. The pesticide propoxur (2-isopropoxyphenyl methylcarbamate) was chosen, as it is widely used for pest control, *e.g.* with carnations.

Recent studies showed that dermal exposure to pesticides in greenhouses is often 100–1000 times higher than inhalatory exposure [1–3]. It remains difficult to estimate health risks for workers caused by dermal exposure because, generally, there is little knowledge on the rate and amount of skin penetration of pesticides under working conditions in greenhouses. For the estimation of health risks, an assessment of the amount of pesticide absorbed is therefore required. Development of biological monitoring methods is, until now, the best strategy in estimating absorbed amounts of pesticides in humans. With biological monitoring it is possible to assess the actual absorbed amount of a compound in the body independent of its route of entrance. To this end, the concentration of the compound or a relevant metabolite in blood or urine is measured. For the interpretation of biological monitoring data, knowledge of the relation between dose and ex-

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cretion is a prerequisite. Therefore a method was needed for the determination in urine of propoxur, or one of its metabolites, that could be applied in laboratory studies with volunteers and subsequently in field studies.

Metabolism studies in rats with intraperitoneally administered radioactive propoxur showed that *ca.* 75% of the radioactivity was eliminated within 24 h as a conjugate of 2-isopropoxyphenol (IPP), probably as a glucuronide [4]. Studies in humans, using oral or inhalatory administration of propoxur, showed that the IPP conjugate was the major urinary metabolite [5,6].

Consequently, IPP was chosen as the metabolite for conducting biological monitoring of propoxur. Analytical methods for IPP in urine, published so far, were all gas chromatographic (GC) methods using packed columns, with or without derivatization [5,7,8]. These methods were either rather laborious [5,8] or did not provide the required detection limit [17]. For the present study, the goal was to develop an analytical method with a detection limit of *ca.* 5 μg/l, if possible without derivatization. This paper describes the development and validation of a gas chromatographic–mass spectrometric (GC–MS) method using single-ion monitoring for the determination of IPP in urine.

EXPERIMENTAL

Chemicals

Analytical-grade hydrochloric acid (37%), *n*-hexane, 2-propanol, disodium hydrogenphosphate and sodium hydroxide were obtained from Merck (Amsterdam, Netherlands). 2-Isopropoxyphenol (97%) and 2,4,6-trimethylphenol (98%) were from Aldrich (Brussels, Belgium). Standard solutions of IPP (10 mg/l) and trimethylphenol (TMP; 40 mg/l) were made in 2-propanol. Buffer was prepared by adjusting 50 ml of 0.05 M Na_2HPO_4 with 0.1 M NaOH to pH 11.

Gas chromatography–mass spectrometry

A Hewlett-Packard Model 5890 gas chromatograph equipped with a Model HP 5970B mass-selective detector and an HP 5970A workstation were used. Samples were injected with an autosampler Model 7673A (Hewlett-Packard, Amstelveen, Netherlands) using splitless injection with a purge off time of 0.75 min. The injection port temperature was 200°C. The temperature of the transfer line was 280°C. Electron impact (EI) ionization was performed at 70 eV with an ion source temperature of 200°C. An OV-1701 fused-silica capillary column, 25 m × 0.25 mm I.D., film thickness 0.2 μm (Chrompack, Middelburg, Netherlands) was used. The column was operated initially at 40°C for 1 min, after which the temperature was increased at 5°C/min to 105°C, and finally to 250°C at 30°C/min for conditioning of the column. The carrier gas was helium with a column inlet pressure of 120 kPa. Data acquisition was started 5 min after injection (solvent delay). Quantification was performed on m/z 110 for IPP (5–12.5 min) and on m/z 136 (12.5–15 min) for TMP. The retention times of IPP and TMP were *ca.* 11.5 and 13.2 min, respectively.

Sample preparation

After thawing and mixing, 4 ml of urine were pipetted into a glass test-tube with ground stopper. Subsequently, 30 μl of a TMP solution (final concentration 300 μg/l of urine) and 150 μl of hydrochloric acid were added and the sample was mixed again. After hydrolysis in a boiling water-bath for 1 h, the samples were cooled to ambient temperature and 3 ml of hexane were added. The urine was extracted for 15 min in a rotary extractor. After centrifugation (15 min at 1800 g), the hexane layer was transferred to another test-tube. The extraction procedure was repeated once. The pooled extracts, *ca.* 6 ml, were washed with 1 ml of phosphate buffer (pH 11) and 1 μl of the hexane layer was used for GC analysis.

Calibration

Calibration curves were made in blank urine. To 4-ml portions of urine, 0, 0.1, 0.2, 0.3, 0.6, 0.8 or 1.0 μg of IPP was added. Subsequently, 1.2 μg of the internal standard TMP were added, and the calibration samples were treated as described above. The calibration curve ranged from 0 to 250 μg/l. The curves were constructed by plotting

the ratio of the peak areas of IPP and TMP against the IPP concentration.

Validation of the method

In order to validate the method with respect to hydrolysis, analytical recovery, limit of detection, stability and precision, urine samples containing IPP conjugates were required. A volunteer (male, body weight 82 kg) was given 7.1 mg of propoxur (dissolved in 75 ml of drinking water). Urine was collected just before dosing and fractionated afterwards, until 48 h after administration. The extraction recovery was determined by spiking urine from non-exposed persons with different concentrations of IPP (29.5, 295 and 368 μg/l) and TMP (297 and 450 μg/l) in duplicate. The recovery was calculated by comparing the peak heights of IPP and TMP after hydrolysis and extraction with those of IPP and TMP directly added to hexane. Buffers of pH 10 (0.05 M NaHCO₃–NaOH), 11 and 12 (0.05 M Na₂HPO₄–NaOH) were tested for their effectiveness in removing interfering compounds from the hexane extract without loss of IPP.

Deconjugation of IPP was optimized with respect to pH and hydrolysis time. Three urine specimens with concentrations of 50, 170 and 350 μg/l IPP, obtained from the pilot study with the volunteer, were selected for this test. To 4 ml of urine, 0.15, 0.5, 1.0 or 1.5 ml of hydrochloric acid were added, and the samples were hydrolysed in a water-bath at 100°C. Free IPP was determined just before hydrolysis and after 10, 30, 60, 90 and 180 min.

Matrix effects were investigated by preparing calibration curves in fourteen different urines from non-exposed persons and comparing the slopes. Three samples from the pilot experiment were used at concentrations of 16, 152 and 219 μg/l to determine the within-day coefficients of variation (C.V.) (n = 6). The stability of IPP conjugates in urine during storage at −20°C and between-day C.V. were determined using the same urines and one extra at a concentration of 29 μg/l.

RESULTS AND DISCUSSION

The mass spectra and structures of IPP and TMP are shown in Fig. 1. The internal standard was necessary to correct for losses during sample clean-up, possible discrimination in the injection port, and fluctuations of the sensitivity of the mass-selective detector. The parent peak in the TMP mass spectrum, m/z 136, was chosen because of interference at m/z 121 in some samples. GC–MS in the single-ion monitoring (SIM) mode was chosen for its specificity and sensitivity. At the start, the base peak from the mass spectrum of IPP, m/z 110, was chosen, and the parent peak from the mass spectrum of the internal standard TMP, m/z 136, using an HP Ultra 2 capillary column (Hewlett-Packard cross-linked 5% phenyl methyl silicone). However, in some urine samples from non-exposed persons, a compound with the same retention time as IPP (11.299 min) was found. This compound was
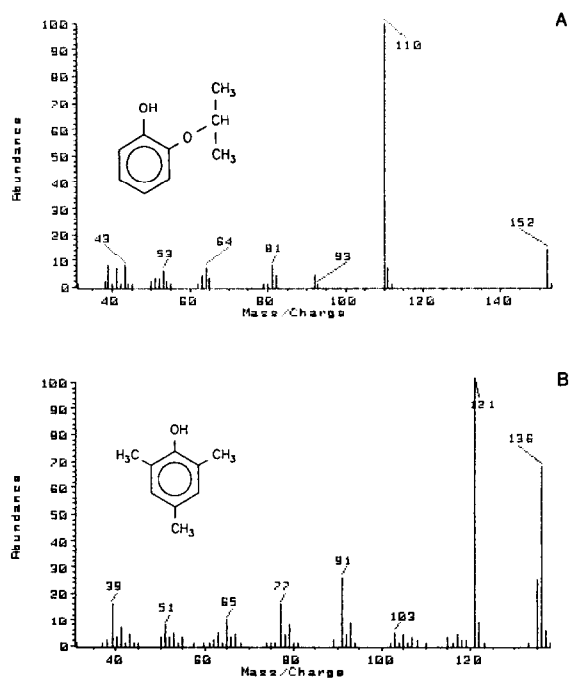

Fig. 1. Mass spectra and structures of 2-isopropoxyphenol (A) and the internal standard 2,4,6-trimethylphenol (B). An Ultra 2 capillary column (25 m × 0.22 mm I.D., film thickness 0.3 μm) was operated at 50°C for 1 min. The temperature was increased by 7.5°C/min to 200°C. Inlet pressure, 89 kPa. Mass spectra were scanned from m/z 30 to 350.
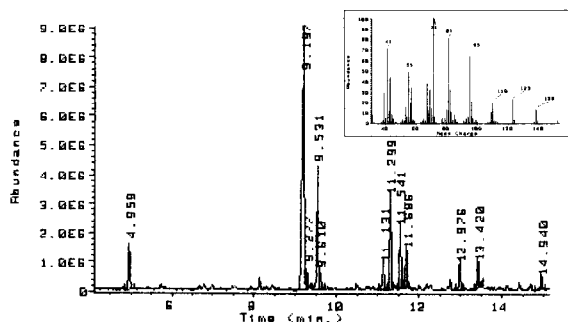
Fig. 2. Total-ion current chromatogram of a blank urine extract. The peak with the same retention time as IPP (11.299 min) was identified as menthol (see inset). Peaks with retention times of 9.197 and 11.131 min are p/m-cresol and an isomer of menthol, respectively. See Fig. 1 for conditions.

Fig. 4. Single-ion monitoring chromatogram of a urine extract of an exposed subject (220 μg/l IPP). The peak with retention time 11.530 min is IPP. The other peaks are menthol (10.839 min), p/m-cresol (M + 2 peak, 11.746 min) and TMP (13.229 min). Conditions: OV-1701 column (see text).

identified as an isomer of menthol, which can originate from chewing gum, toothpaste or menthol cigarettes. Two menthol isomers with retention times of 11.131 and 11.299 min could be identified in these extracts (Fig. 2). This problem could be eliminated by either monitoring another ion of the IPP spectrum (m/z 152), which implies a five-fold decrease in abundance, or by changing the capillary column. We chose to replace the Ultra 2 column by a more polar OV-1701 column, using the same conditions for the mass-selective detector. Menthol then no longer interferes with the quantification of IPP (Fig. 3). A representative chromatogram of a urine extract from an exposed person is shown in Fig. 4.
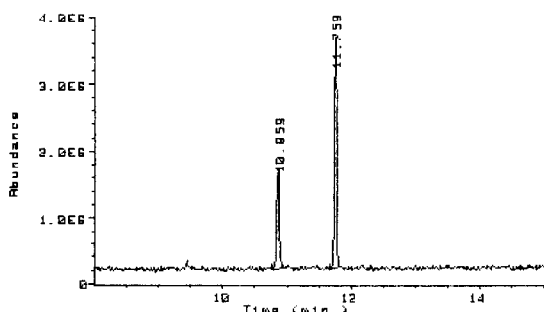
The extraction recovery of IPP and TMP from spiked urine after hydrolysis (pH 1), extraction and clean-up was better than 95%. Phosphate buffer (pH 11) was used to clean up the hexane

TABLE I

ANALYTICAL RECOVERY OF IPP AND TMP

Analytical recovery of spiked IPP and TMP from blank urines after hydrolysis (pH = 1) and extraction at different concentrations. Cleaning up was tested at pH 10, 11 and 12. Recovery was calculated by comparing the peak heights of IPP and TMP with those of IPP and TMP directly added to hexane.

IPP (μg/l)	TMP (μg/l)	Recovery (%)
pH 1		
29.5		96/96
295		95/93
368		93/95/97/98
	297	93/92/95/93
	450	109/113
pH 10		
295		101/98
	450	104/100
pH 11		
29.5		97/96
295		93/93
368		101/98
	297	93/92/93/94
	450	105/98
pH 12		
295		66/70
	450	87/88

Fig. 3. Total-ion current chromatogram of a blank urine extract. Peaks with retention times of 10.859 and 11.759 min were menthol and p/m-cresol, respectively. No peak was detected with the same retention time as IPP (11.5 min). Conditions: OV-1701 column (see text). Mass spectra were scanned from m/z 30 to 350.
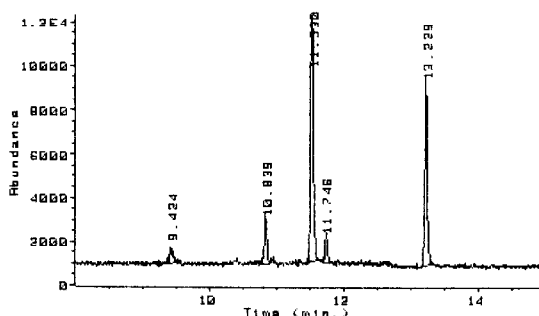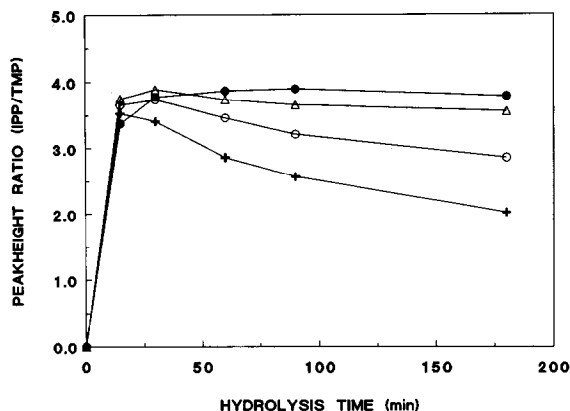
Fig. 5. Hydrolysis of 4 ml of urine containing 350 μg/l conjugated IPP with 0.15 (●), 0.5 (△), 1.0 (○) or 1.5 (+) ml of hydrochloric acid.

extracts. This pH was most effective, with a minimum loss of IPP and TMP. At a pH of 12 considerable loss of IPP was observed (Table I).

When hydrolysing urine samples from the pilot experiment with different amounts of hydrochloric acid, we observed an increase in the free IPP concentration followed by a decrease during hydrolysis when 0.5 ml or more of hydrochloric acid was added (Fig. 5). With 150 μl of hydrochloric acid (urine pH *ca.* 1) we were able to hydrolyse the conjugates within 30 min, after which free IPP appeared to be stable. In order to be on the safe side, hydrolysis was performed for 60 min. From Fig. 5 one can also infer that IPP is excreted fully conjugated in urine. Without hydrolysis no IPP was detected.

Calibration curves were made in blank urines

TABLE II

WITHIN-DAY AND BETWEEN-DAY VARIATION FOR THE DETERMINATION OF IPP IN URINE

The number of samples is given in parentheses.

Control sample	Within-day variation (mean ± S.D.) (μg/l)	Between-day variation (mean ± S.D.) (μg/l)
1	16 ± 1.1 (6)	15 ± 3.1 (33)
2	152 ± 3.5 (6)	150 ± 10.9 (37)
3	219 ± 3.9 (6)	213 ± 9.1 (35)
4		29 ± 2.8 (27)

from fourteen different subjects and showed excellent linearity up to concentrations of 250 μg/l ($r = 0.9984$, S.D. = 0.0009). The slopes of these curves were 0.0107, with an S.D. of 0.0009. From the S.D. we concluded that no matrix effect occurred. No peaks were observed with the same retention times as IPP and TMP in urine from non-exposed persons. The limit of detection, defined as three times the signal-to-noise ratio, was *ca.* 6 μg/l. The IPP conjugates were stable for at least seven months when stored at $-20°C$. The within-day C.V. ($n = 6$) were 7, 2 and 2% at concentrations of 16, 152 and 219 μg/l, respectively. The between-day C.V., as calculated from the results of the stability test, were 20, 10, 7 and 4% at 15 ($n = 33$), 29 ($n = 27$), 150 ($n = 37$) and 213 μg/l ($n = 35$), respectively (Table II).

In the subject who received an oral dose of propoxur, *ca.* 80% of the dose was excreted as IPP (Fig. 6). This percentage was representative for other volunteers we examined. Preliminary results of these studies with propoxur will be published elsewhere [9]. Only one study reported the excretion of IPP after oral administration of propoxur in humans [5]. In this study, three males took 50 mg, after which an average of 27% was recovered as IPP in urine. Possible explanations for the lower values obtained by Dawson *et al.* [5] may be the different protocol used for the administration of propoxur and collection of urine samples, or the loss of IPP during hydrolysis.
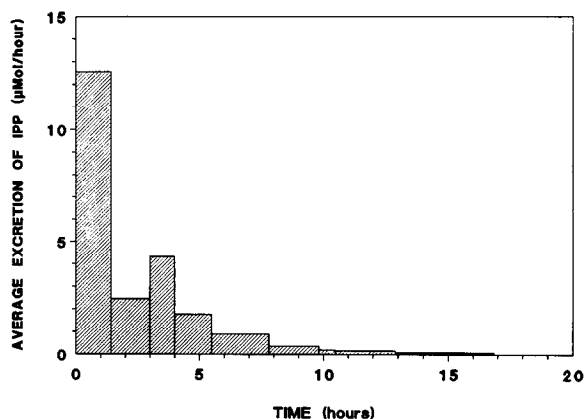

Fig. 6. Average excretion of IPP in urine after an oral dose of 7.1 mg of propoxur (0.086 mg/kg, male, 82 kg).

The accuracy of the method cannot be fully evaluated because no external quality control samples are available for IPP conjugates. However, the facts that, on average, 80% of an orally administered dose of propoxur is recovered as IPP and that no IPP is detected in the urine of non-exposed persons, indicate that all conjugated IPP is liberated after hydrolysis.

CONCLUSION

GC with mass-selective detection offers a suitable analytical method for the determination of low concentrations of IPP in urine. This method can thus be used in studies with volunteers in order to develop a validated method for the biological monitoring of propoxur. Hydrolysis of IPP conjugates appeared to be a critical step in this assay, where pH and duration should be controlled carefully. The hexane extracts are used directly for the quantitation of IPP. Lower detection levels can be achieved by concentrating the extracts under nitrogen. This, however, requires the use of a keeper in order to prevent evaporation of IPP.

ACKNOWLEDGEMENT

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