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Gas-chromatography mass-spectrometry determination of phthalic acid in human urine as a biomarker of folpet exposure

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Abstract

Agricultural workers are exposed to folpet but biomonitoring data are limited. Phthalimide (PI), phthalamic acid (PAA) and phthalic acid (PA) are the ring metabolites of this fungicide according to animal studies but they have not yet been measured in human urine as metabolites of folpet, only PA as a metabolite of phthalates. The objective of this study was thus to develop a reliable gas chromatography – tandem mass spectrometry (GC-MS) method to quantify the sum of PI, PAA and PA metabolites ring-metabolites of folpet in human urine. Briefly, the method consisted of adding *p*-methylhippuric acid as an internal standard (IS), performing an acid hydrolysis at 100°C to convert ring-metabolites into PA, purifying samples by ethyl acetate extraction and derivitazing with N,O-bis(trimethylsilyl)trifluoro acetamide (BSTFA) prior to GC-MS analysis. The method had a detection limit of 60.2 nmol/L (10 ng/mL); it was found to be accurate (mean recovery of 97%), precise (inter- and intra-day percentage relative standard deviations < 13%) and with a good linearity ($R^2 > 0.98$). Validation was conducted using unexposed person urines spiked at concentrations ranging from 4.0 to 16.1 µmol/L, along with urine samples of volunteers dosed with folpet and of exposed workers. The method proved to be i) suitable and accurate to determine the kinetic profile of PA equivalents in the urine of volunteers orally and dermally administered folpet and ii) relevant for the biomonitoring of exposure in workers.

Keywords Phthalic acid · Phthalamic acid · Folpet · Human urine · Biological monitoring

Introduction

Folpet, or N-trichloromethyl-thio-phthalimide (CAS number 133-07-3), has extensive application as a contact fungicide in a variety of fruit and vegetable crops or vineyards. Although a large number of workers use or are in contact with folpet, there is a paucity of data on the extent of occupational exposure to this compound. Some environmental measurements have been conducted [1] but only one study performed a biomonitoring of exposure to folpet in pregnant women through phthalimide (PI) measurements [2].

Folpet metabolism is however well characterized in animals and *in vitro* studies [3-7]. According to these studies, folpet is rapidly metabolized to PI and thiosphogene, an unstable metabolite which reacts with cysteine or gluthathione to form thiazolidine-2-thione-4-carboxylic acid (TTCA). Phthalimide is also rapidly hydrolyzed to phthalamic acid (PAA), and in turn to phthalic acid (PA). The chemical structures of folpet and of its ring metabolites are displayed in Fig. 1.

According to Chasseaud *et al.* [4;5;8] and Canal-Raffin *et al.* [3], the main ring-metabolite of folpet is PAA in rats following an oral, intratracheal or intraperitoneal administration. More specifically, Chasseaud *et al.* [5;8] observed that 80% of a labeled ^{14}C-folpet dose orally administered to rats was recovered as PAA in urine. Canal-Raffin *et al.* [3] reported that PI was rapidly metabolized to PAA in plasma when folpet was intraperitoneally and intratracheally administered to rats.

To quantify PAA, Canal-Raffin *et al.* [3] analyzed rat plasma by high-performance liquid chromatography with UV detection (HPLC-UV) after specific solid-phase extraction, but they

did not measure the metabolite in urine. Nevertheless, as determined by Bray *et al.* [9], PAA is a very unstable compound in urine, which has to be precisely extracted at pH 8.3 and is transformed to PA in acidic conditions (pH 2-3). Namely, under electron impact ionization (EI) for GC-MS analysis, water loss is induced and the neutral compound PAA can rearrange to PI, phthalic anhydride or phthalic acid [10;11]. It is thus more convenient to transform PI and PAA metabolites of folpet to PA in acid conditions, and to measure total PA equivalents in urine as a biomarker of exposure.

Analytical methods have already been developed to quantify PA as a urinary metabolite of phthalates, because it is also the final hydrolysis product of phthalates [12-16], but no method has been published for the quantification of total PA equivalents as a biomarker of folpet exposure to our knowledge. Therefore, the objectives of this study were i) to measure total PA equivalents in urine as a biomarker of folpet exposure by adapting existing gas chromatography - mass spectrometry [GC-MS] methods after oxidation of PI and PAA and trimethylsylation of PA molecule, and ii) to use this method to quantify the urinary excretion of total PA equivalents in volunteers exposed to folpet as well as iii) to assess worker exposure to this fungicide through biomonitoring.

Materials and methods

Chemicals and reagents

Phthalic acid (1,2-Benzenedicarboxylic acid), phthalamic acid (benzoic acid,2-aminocarbonyl), phthalimide and *p*-methylhippuric acid used as reference standards (>99% purity) were obtained from Sigma-Aldrich (Buchs, St Gallen, Switzerland). Other chemicals and reagents such as HPLC-grade acetonitrile, dimethylformamide, ethyl acetate, N,O-bis(trimethylsilyl)trifluoro acetamide (BSTFA), hydrochloric acid and sodium chloride (NaCl) were also purchased from Sigma-Aldrich (Buchs, St Gallen, Switzerland). Purified tap water by a TKA GenPure (Niederelbert, Germany) was used.

Standard preparation

Stock standard solutions

A stock standard solution of 130 mg/L (0.8 mmol/L) of PA was prepared by dissolving 13 mg of PA in 100 mL of 1% (v/v) dimethylformamide in acetonitrile. Similarly, a stock standard solution of the internal standard (IS) *p*-methylhippuric acid of 100 mg/L (0.5 mmol/L) was prepared by dissolving 10 mg of *p*-methylhippuric acid in 100 ml of 1% (v/v) dimethylformamide in acetonitrile. In addition, to measure the fraction of PI and PAA converted to PA with this analytical method, two individual stock standard solutions of 112 mg/L (0.8 mmol/L) of PI and 114 mg/L (0.7 mmol/L) of PAA were prepared by dissolving 11.2 mg of PI and 11.4 mg of PAA, respectively, in 100 mL of 1% (v/v) dimethylformamide in acetonitrile. The stock solutions were stored at -20°C in glass containers until used.

Calibration curves

Calibration curves were prepared by spiking aliquots of pooled urine from unexposed persons at different concentrations of PA and a constant concentration of IS. Working solutions of PA at 26 mg/L (0.16 mmol/L) and IS at 20 mg/L (0.1 mmol/L) were thus prepared daily from stock solutions diluted 5-fold in water. Calibration curves of PA consisted of urine from unexposed persons spiked at six concentration levels prepared in duplicates: 0, 0.67, 1.3, 2.0, 2.3 and 2.7 µg/mL (0, 4.0, 8.1, 12.1, 14.1 and 16.1 µmol/L). For each level, 2 mL of urine were spiked with a constant volume of 100 µL of IS working solution as well as defined volumes of PA working solution and completed with water to obtain a final volume of 2.3 mL.

Sample treatment

The analytical approach used to quantify total urinary PA equivalents consisted of an acid hydrolysis, solvent extraction and derivatization with BSTFA followed by gas chromatography – mass sprectrometry (GC-MS) analysis. Sample preparation was adapted from method of Mettang *et al.* [14]. Two-mL aliquots of urine were transferred into screw-cap reaction vials and spiked with 100 µL of internal standard working solution (20 mg/L or 0.1 mmol/L) and 200 µL of water. An acid hydrolysis was then performed by adding HCl (300 µL at 12 N) and heating at 100°C for 12 h. After cooling, samples were extracted twice with 2 mL of ethyl acetate by agitating for 15 min and centrifuging for 10 min at 2500 rpm. Upper organic layers were transferred into glass tubes. Extracts were evaporated to dryness under a gentle nitrogen flow at 40°C to concentrate samples. Residues were resuspended in 300 µL of ethyl acetate; 30 µL of BSTFA were added and samples were heated at 60°C for 60 min to convert total PA equivalents and *p*-methylhippuric acid IS into trimethylsilyl phthalic acid (TMS-PA) and trimethylsilyl *p*-methylhippuric acid (TMS-IS), respectively, by replacement

of the labile hydrogen of the hydroxy groups. Before transferring derivatized extracts to vials for GC-MS analysis, extracts were cooled for a 2-h period.

Instrumental analysis

Analyses were carried out on a HP 5973 gas chromatograph equipped with a mass selective detector Agilent MSD-G1098A and a CP-SIL 8 CB fused silica capillary column (60 m length, 1 μm film thickness, 250 μm I.D.). Helium was used as a carrier gas at a 27 cm/s linear velocity. The injector was set to a temperature of 260°C and a constant column flow rate was held at 1.0 mL He/min. The transfer line was set at 250°C and the ion source at 230°C. The GC oven temperature program was set initially at 200°C for 3 min; it was then increased to 260°C at 30°C/min, held for 11 min, and finally increased to 280°C at 35°C/min and held for 4 min. The mass detector was operated in single ion monitoring mode for quantification and fragment ions were generated by electron impact ionization at 70 eV. The fragments analyzed were *m/z* 295 for TMS-PA (qualitative ions were *m/z* 221 and 147) and *m/z* 220 (qualitative ions were *m/z* 177 and 119) for TMS-IS. Two μL were injected onto the GC with an Agilent auto-sampler using a 5 mL/min split. Under these conditions, retention times were 10.6 min for TMS-PA and 16.0 min for TMS-IS.

Quantification of PA

Quantification of PA was carried out using standard calibration curves in urine. This was achieved by plotting the response factors as a function of the six standard concentration levels of PA (0, 0.67, 1.3, 2.0, 2.3 and 2.7 μg/mL or 0, 4.0, 8.1, 12.1, 14.1 and 16.1 μmol/L). The response factors were the peak-height ratios of TMS-PA to TMS-IS.

Estimation of the fraction of PI and PAA converted to PA

The fraction of PI and PAA converted to PA with the analytical processing used was also determined. Aliquots of urine from an unexposed person were independently spiked with standard solutions of 22.5 mg/L of PI or 22.8 mg/L of PAA (stock standard solutions diluted 5-fold) at six concentration levels prepared in duplicates: 0, 0.56, 1.12, 1.69, 1.97 and 2.25 g/L (0, 3.8, 7.6, 11.5, 13.4 and 15.3 mmol/L) for PI and 0, 0.57, 1.14, 1.71, 1.99 and 2.28 g/L (0, 3.4, 6.9, 10.3, 12.1 and 13.8 mmol/L) for PAA. These samples were then treated using the method described previously for the quantification of TMS-PA. The fraction of PI and PAA converted to total PA equivalents (expressed as a percentage) was calculated using the following equation:

$$\text{Fraction of PI and PAA converted to PA (\%)} = \frac{\text{Amounts of PA in extracts of spiked samples (mol)}}{\text{Added amounts of PI or PAA in urine samples (mol)}} \times 100$$

Method validation

In order to validate the method, the following criteria were verified: limit of detection (LOD), limit of quantification (LOQ), linearity, intra- and inter-day precision, accuracy, recovery and stability. In addition, internal quality control (QC) urinary samples were prepared from a pool of urine from unexposed individuals spiked at two levels of concentrations (1 and 2.5 µg/mL or 6 and 15 µmol/L). From this pool, 6-mL aliquots were prepared and stored at -20°C. During each daily run, QC samples were analyzed in triplicates, as unknown samples.

Specificity of the method was confirmed by verifying the absence of interferences on the chromatograms obtained from urine samples of unexposed individuals (n = 22). The LOD was established as three times the signal-to-noise ratio for the specific ions *m/z* 295 and *m/z* 220 and the LOQ as ten times the signal-to-noise ratio. Linearity was calculated from

regression parameters of 22 calibration curves, by the least square fit method. Results were expressed using the mean of coefficient of determination (R^2).

To assess intra- and inter-day variations, the precision and accuracy were calculated from replicate analysis of previously prepared QC samples and of daily prepared aliquots of a pool of urine from unexposed individuals spiked with PA at five different levels. The intra-day variation was estimated by the repeatability of triplicates of QC samples and of duplicates of each spiking level prepared daily and analyzed the same day. The inter-day variation was estimated by the reproducibility of these previous samples on 22 consecutive days.

Precision, expressed as percentage of relative standard deviation (% RSD), was determined as the ratio of standard deviation to mean of the response factor for each spiking level (QC samples and prepared daily spiked samples) multiplied by 100. Accuracy, expressed in percentage, was calculated as follows:

$$\frac{(\text{average measured amounts of PA} - \text{known spiking amounts of PA})}{\text{known spiking amounts of PA}} \times 100$$

Recovery of PA in urine after extraction and derivatization was calculated for each spiking level by the ratio of measured amounts of PA in extracts to the theoretical spiked amounts in urine of unexposed individuals and expressed as a percentage.

The stability of PA in urine was ascertained by analyzing every day over a 22-day period an aliquot of QC samples kept at -20^{o}C, and thawed daily and processed. The stability of PA equivalents in urinary samples of workers exposed to folpet was also determined. More specifically, the effect of preservation of urine samples at 4^{0}C over a 24-h-period was tested.

Two urine samples of workers thawed, processed and analyzed for PA equivalents in a given run were thus kept at 4°C, processed and reanalyzed during the following run.

Application of the method

The method was used to determine total urinary amounts of ring-metabolites of folpet in exposed individuals. First, concentrations of total PA equivalents were determined in all urines collected repeatedly and at predetermined times over a 96-h period following an oral and dermal administration of 1 and 10 mg/kg of folpet, respectively, in five volunteers (the same five volunteers were used for the oral and dermal dosing).

Secondly, concentrations of PA equivalents were measured in all complete urine voids collected over a 7-day period following a folpet treatment and sequences of harvesting in grapegrowers (n = 3). Each worker collected i) a pre-seasonal complete first-morning void, hence a urine sample prior to folpet spraying and harvesting season, ii) all complete urine voids prior to and during a typical workweek following a folpet spraying, and iii) all complete urine voids prior to and during a typical workweek involving harvest activities. During spraying period, the three workers applied folpet only on the first workday; during harvest activities period, workers 1 and 3 were in the treated area only on workday 1 while worker 2 harvested from workdays 1 to 3.

The Permanent Ethical Committee of Clinical Research of the Faculty of Biology and Medicine of the University of Lausanne and of the Research Ethical Committee of the Faculty of Medicine of the University of Montreal approved the protocol, and all participants gave their written informed consent, and were acquainted with the risks of participating and their right to withdraw from the study at all time. The volunteers administered folpet received a

small monetary compensation for their time and any inconvenience caused, as suggested by the Ethics Committee who considered the study as restrictive. Conversely, workers were not compensated.

Results and discussion

Method development

To optimize the method of Mettang *et al.* [17] for the specific analysis of total PA equivalents as a biomarker of exposure to folpet, several preliminary tests were performed: 1) the verification of potential contamination of laboratory materials by phthalates, 2) the selection of a proper IS, 4) the effect of NaCl addition prior to extraction and 4) the establishment of the most efficient type of hydrolysis. Firstly, to verify if that laboratory materials (i.e. vials, caps, pipettes) were a contamination source of PA during sample processing, triplicate controls with water and ethyl acetate were subjected to the processing procedure; no PA peak was observed after analysis. Secondly, potential internal standards were also tested. Four deuterium phthalic acids (PA-d) were initially considered, but the separation of PA and PA-d on the GC column was insufficient to differentiate both peaks on chromatograms. *Para*-methylhippuric acid was then assayed because the molecule comprised a carboxylic acid and an amine group, and finally selected. Thirdly, to improve extraction, the effect of adding NaCl to urine samples prior to extraction and analysis was compared with results obtained after analysis of the same urine samples without NaCl addition. The results are presented in Table 1 and show that extraction efficiency was better without NaCl addition.

Lastly, efficiency of acid hydrolysis as compared to enzymatic hydrolysis was tested. This latter step was described by several authors for the quantification of phthalates [14;17-23]. To perform enzymatic hydrolysis, 20 µL of β-glucuronidase-arylsulfatase were added to 2 mL of four urine samples from one person administered folpet and samples were heated at 37°C for 12 h; for acid hydrolysis, 500 µL of HCl 2N were added and samples were heated either at 37°C or 100°C for 12 h. As shown in Table 2, higher concentrations of total PA equivalents

were observed after acid hydrolysis at 100°C than at 37°C, while enzymatic hydrolysis appeared inadequate and insufficiently strong to transform PI and PAA into PA. Pfäffli [24] previously reported that enzymatic hydrolysis was unnecessary because PA appears to be mainly excreted as free acid and not partly conjugated and excreted as glucuronide. Table 3 also shows that when spiking urine samples with PAA and PI, PAA is virtually all converted to TMS-PA (100%) and half of PI was converted to TMS-PA (≈50%) following an acid hydrolysis at 100°C. Thus, acid hydrolysis was selected for the quantification of total PA equivalents. Even if PI was not completely converted to TMS-PA by acid hydrolysis, it is a very minor metabolite of folpet as compared to PAA according to *in vivo* studies in rats exposed to folpet [3-8].

Representative chromatograms of TMS-PA and TMS-IS in urine from an unexposed individual spiked with 12.1 µmol/L of PA and 6.72 µmol/L of IS, as well as the mass spectra and the molecule structure of both derivatives are presented in Figure 2. These chromatograms were obtained with the optimized method after acid hydrolysis, solvent extraction and derivatization. Chromatograms show very limited background interference. Clean chromatograms were observed not only for spiked and non-spiked urines from unexposed individuals, but also for samples from volunteers dosed with folpet and from workers, as displayed in Fig. 3. Therefore, analytical conditions were specific to quantify TMS-PA as the sum of total ring-metabolites folpet.

It is to be noted that TMS-PA was detectable in urine samples of unexposed persons, as illustrated in Fig. 3b. Hence, an average PA concentration (± SD) of 285 ± 150 nmol/mmol creatinine (492 ± 275 ng/mL) was obtained in the urine of unexposed individuals (n = 10) and 186 ± 79.7 nmol/mmol creatinine (507 ± 280 ng/mL) in pre-seasonal urines of workers

exposed to folpet (n = 3). These values are equivalent to PA concentrations determined by Pfäffli [24] in pre-shift urines of workers exposed to phthalic anhydride (490 nmol/mmol creatinine) and in urines of occupationally unexposed people (0.34 µmol/mmol creatinine). Similarly, in biomonitoring studies of phthalates, Jönsson *et al.* [25], Kato *et al.* [19] and Vermeulen *et al.* [26] reported also relatively high PA baseline levels in unexposed people or in workers during a non-exposure period. These levels were on average lower than those found in our study; however, large interindividual variability was found. Namely, Jönsson *et al.* [25] reported a mean PA concentration of 94 nmol/mmol creatinine (95^{th} percentile) in urine samples from 234 Swedish men, with a maximum concentration of 2100 nmol/mmol creatinine; Kato *et al.* [19] measured a median PA concentration of 6.19 ng/mL in 43 unexposed and anonymous adults with a range of 1.54 to 44.4 ng/mL (creatinine was not measured in this study); Vermeulen *et al.* [26] obtained a median PA concentration of 77 ng/mL (47 nmol/mmol creatinine) in Sunday urine samples of rubber workers with a range of 4 to 2449 ng/mL (2.44 to 1495 nmol/mmol creatinine). This baseline is unavoidable because phthalates are ubiquitous in the environment and exposure can occur from various sources [12;14-31]; exposure to folpet in the general population can also occur through the diet, thus also contributing to baseline levels. Biomonitoring results should thus be interpreted with caution in non-occupationnally exposed invididuals. However, these studies observed higher PA concentrations when people were occupationally exposed to phthalates.

Method validation

To validate the robustness of the current method, we evaluated its linearity, LOD, LOQ, intra- and inter-day precision and accuracy, recovery and stability using a pool of urine from unexposed individuals spiked at five different levels and two QC samples.

All calibration curves (n = 22) were linear within the spiking range in urine of 0.67 to 2.7 µg/mL (4.0 to 16.1 µmol/L) with a mean equation of $y = 0.957x + 0.088$ and a coefficient of determination of 0.98. The LOD and LOQ of PA in urine were 10 ng/mL (60.2 nmol/L) and 33 ng/mL (199 nmol/L), respectively. In comparison with methods developed for the analysis of PA as a biomarker of phthalate exposure, our method had a better LOD than the one obtained with the liquid chromatography – tandem mass spectrometry (LC/MS/MS) method described by Jönsson *et al.* [25] (LOD = 15 ng/mL or 90.3 nmol/L) or the GC-MS method of Pfäffli [24] (LOD = 100 ng/mL or 602 nmol/L) and a LOD similar to the one reported by Lim *et al.* [13] with a HPLC/UV method (LOQ = 100 ng/mL or 602 nmol/L). However, Kato *et al.* [19], Silva *et al.* [22] and Vermeulen *et al.* [26] reported LC/MS/MS methods for the quantification of PA metabolites of phthalates with a slightly better LOD (LOD of 0.42, 1.59 and 5 ng/mL or 2.53, 9.57 and 30.1 nmol/L, respectively). Nonetheless, to determine phthalate exposure to date, specific metabolites (i.e. monoesters and oxidation products) are preferably measured instead of PA, which is a common final metabolite of many phthalates [13; 17-18; 28-29].

Results for precision and accuracy of the method are summarized in Table 4 for each of the five daily prepared spiking levels of aliquots of a pool of urine from unexposed individuals as well as for two pre-prepared QC samples. Results indicate that urinary PA was reliably measured in urine because the intra- and inter-day precision expressed by the percent relative standard deviation (% RSD) was less than 13% overall (less than 10% for spiking amounts higher than 2 nmol), and accuracy established by the percent relative error (% RE) was less than 10%.

Table 4 also reveals an excellent recovery of PA in extracts after acid hydrolysis, solvent extraction and derivatization of urine samples spiked with PA. Hence, the mean (± SD) recovery of PA (all spiked levels combined) was 97.5 ± 8.8%, indicating that very little PA was lost during processing.

QC samples kept at -20^{o}C and thawed daily and processed over a 22-day period also appeared stable; this is evident when comparing mean intra-day variation in recovered PA amounts to mean inter-day variation (Table 4). Likewise, PA was stable in urine samples of workers exposed to folpet. A coefficient of variation ranging from 6.9 to 13.3% was obtained when two aliquots of the same urine collection of two workers kept at 4^{o}C for 24 h were processed and analyzed during the following run.

Application of the method to biomonitoring

To further validate the method and verify that total ring-metabolites of folpet (*i.e.* total PA equivalents) could be efficiently quantified in the urine of individuals exposed to folpet, urines of volunteers orally and dermally dosed with folpet as well as urines of grapegrowers during a typical work week were analyzed.

The mean concentration-time profiles of total PA equivalents in the urine of volunteers following an oral and dermal administration are presented in Fig. 4. Although there was a background PA level, it was easy to document the human toxicokinetics of this biomarker of exposure [32-33]. Briefly, PA had a relatively short elimination half–life in urine and it was similar for both exposure routes, hence 27.6 h and 29.6 h for oral and dermal routes, respectively. Similarly, Table 5 displays pre- and post-shift urinary levels of total PA equivalents in workers exposed to folpet during and following spraying or harvest activities.

PA was measurable in all urine samples of workers and an increase in excretion was observed for most workers following spraying or harvest activities. According to these results, workers were more exposed during spraying activities than during harvest activities. Consequently, the method proved to be suitable to quantify actual daily exposures to folpet in workers.

Conclusion

In summary, the method was found to have a LOD of 60.2 nmol/L, an excellent recovery (mean of 97%), a good linearity with a R^2 superior to 0.98. It is also precise with an inter- and intra-day percentage relative standard deviations inferior to 13%. In addition, with this method, only a small urine volume is needed (2 mL) and sample treatment is straightforward. Moreover, our results evidenced that the current GC-MS method was reliable and accurate to quantify total PA equivalents the in urine of individuals dosed with folpet as well as relevant to assess worker exposure using biomonitoring.

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Table 1

Comparison of PA concentration (nmol/mL) measured in four urine samples collected at different time points in a same individual experimentally administered folpet by the oral route, with or without the addition of sodium chloride prior to extraction.

Urine sample[b]	PA concentrations (nmol/mL)[a]	
	Extraction	
	without NaCl	with NaCl[c]
Urine 1	4.60	3.91
Urine 2	33.6	27.2
Urine 3	122	103
Urine 4	36.8	35.4

[a] Urinary concentrations observed following sample processing as described in Materials and Methods but with or without addition of sodium chloride prior to extraction.

[b] Urine samples collected at four different time periods in one volunteer orally administered folpet (1 mg/kg dose).

[c] 250 mg of sodium chloride (NaCl) were added to 2 mL of urine prior to first extraction.

Table 2

Comparison of PA concentration (nmol/mL) measured in four urine samples collected at different time points in a same individual experimentally administered folpet by the oral route following an enzymatic hydrolysis at 37°C or an acid hydrolysis at 37°C or 100°C.

Urine sample[b]	PA concentrations (nmol/mL)[a]		
	Enzymatic Hydrolysis[c]	Acid hydrolysis at 37°C[d]	Acid hydrolysis at 100°C[e]
Urine 1	2.15	2.26	4.03
Urine 2	2.62	12.5	13.7
Urine 3	2.62	16.5	17.2
Urine 4	2.82	24.2	24.3

[a] Urinary concentrations observed following sample processing as described in Materials and Methods but with either enzymatic hydrolysis at 37°C or acid hydrolysis at 37°C or 100°C.

[b] Urine samples collected at four different time periods in one volunteer orally administered folpet (1 mg/kg dose). Urines were different from those used to test the effect of addition of NaCl on extraction efficiency (Table 1).

[c] 20 µL of glucuronidase-arylsulfatase were added to 2 mL of urine and samples were heated at 37°C for 12 h.

[d] 500 µL of HCl 2N were added to 2 mL of urine and samples were heated at 37°C for 12 h.

[e] 500 µL of HCl 2N were added to 2 mL of urine and samples were heated at 100°C for 12 h.

Table 3

Conversion of PAA and PI as TMS-PA (expressed as a percentage) after sample processing (acid hydrolysis, extraction and derivatization) of aliquots of a same urine from an unexposed subject spiked at five different levels (nmol added).

Spiked metabolite	Amount added[a] (nmol)	Amount of TMS-PA found[b] (nmol)	Conversion[c] (%)
PAA	6.90	6.42	93.1
	13.8	14.3	104
	20.7	20.7	99.8
	24.1	24. 6	102
	27.6	27.9	101
PI	7.65	4.79	62.6
	15. 3	7.99	52.3
	22.9	10.3	44.9
	26.8	13.2	49.5
	30.6	16.4	53.5

PAA : phthalamic acid ; PI : phthalimide ; TMS-PA : trimethylsilyl-phthalic acid.

[a] Amount of PAA or PI (expressed in nmol) added to the urine of an unexposed subject and processed as described in Materials and Methods. Duplicates were prepared for each spiking level.

[b] Amount of total PA equivalents recovered (as TMS-PA) after sample processing of a urine spiked with PAA or PI and after subtracting baseline amounts of PA found in urine of the unexposed subject, which was 0.6025 nmol (calculated from the ratio of the intercept to slope of the calibration curve).

[c] Percent of PAA or PI converted to TMS-PA after sample processing, as described in Materials and Methods, of a urine spiked with different amounts of PAA and PI.

Table 4

Recovery of PA, intra- and inter-day precision and accuracy from replicate analysis of daily prepared aliquots of a pool of urine from unexposed individuals spiked with PA at five different levels and previously prepared QC samples spiked at two levels (n = 20 replicates for each spiking level).

			Intra-day variation[a]				Inter-day variation[b]			
Amount added (nmol)[c]	Recovery[d] (%)	RSD[e] (%)	Amount found[f] (nmol) (mean ± SD[g])	Precision (% RSD[e])	Overall RSD[h] (%)	Accuracy[i] (%)	Amount found[f] (nmol) (mean ± SD[g])	Precision (% RSD[e])	Overall RSD[h] (%)	Accuracy[i] (%)
Spiked urine from unexposed persons										
1.33	95.8	11.3	1.28 ± 0.14	11.3	8.65	–4.22	1.28 ± 0.11	8.33	6.09	–4.00
2.66	99.5	9.04	2.65 ± 0.24	9.04		–0.48	2.66 ± 0.18	6.65		–0.08
3.99	97.6	7.08	3.90 ± 0.28	7.08		–2.41	3.90 ± 0.21	5.39		–2.27
4.66	96.3	8.28	4.49 ± 0.37	8.28		–3.73	4.49 ± 0.28	6.31		–3.65
5.33	98.6	9.29	5.25 ± 0.49	9.29		–1.41	5.24 ± 0.31	5.99		–1.64
QC spiked samples										
1.98	91.9	12.5	1.83 ± 0.23	12.5	10.14	–8.06	1.83 ± 0.16	8.64	6.87	–7.94
4.96	98.2	9.26	4.91 ± 0.45	9.26		–1.18	4.91 ± 0.32	6.51		–1.16

[a] Average variation between *N* replicates of the same level of spiking prepared and analyzed the same day.

[b] Average variation between *N* replicates of the same level of spiking prepared and analyzed on different days over a 22-day period.

[c] Amount of PA (expressed in nmol) added at the different concentration levels to aliquots of a pool of urine from an unexposed individual.

[d] Percent recovery of total PA amounts as TMS-PA (different levels) after addition of PA to urine from an unexposed individual and processed as described in Materials and Methods.

[e] Precision or RSD: relative standard deviation for 20 replicates.

[f] Recovered amount of total PA (as TMS-PA) after sample processing and calculated from calibration curves by subtracting baseline amounts of PA found in urine of the unexposed subject (calculated from the ratio of the intercept to slope of the calibration curve).

[g] SD: standard deviation of *N* replicates.

[h] Overall RSD: mean relative standard deviation with all spiked urine from unexposed persons combined (n = 100) and all QC samples combined (n = 40).

[i] Accuracy, or percent relative error.

Table 5

Creatinine adjusted urinary concentrations of total PA equivalents in workers during the course of a typical workweek following a spraying episode of folpet or harvest activities in a previously treated area, along with pre-seasonal concentrations.

				Urinary PA concentration (nmol/mmol creatinine)									
Workers[a]	Activities[b]	Shift Length[c]	Pre-seasonal	Workday 1[d]		Workday 2		Workday 3		Workday 4		Workday 5	
				Pre-shift	Post-shift	Pre-shift	Post-shift	Pre-shift	Post-shift	Pre-shift	Post-shift	Pre-shift	Post-shift
Worker 1	Spraying	4 h	268	655	2448	1152	2790	1557	3508	1252	1528	1120	1779
	Harvest	6 h	268	846	1244	1293	1486	913	2496	1604	ns	ns	ns
Worker 2	Spraying	6 h	108	152	312	588	561	636	435	195	162	135	246
	Harvest	36 h	108	237	230	197	256	345	234	311	259	598	214
Worker 3	Spraying	3 h	181	334	845	406	334	357	174	312	242	227	292
	Harvest	38 h	181	126	216	219	155	130	409	275	771	310	124

ns: no sample collected.

[a] Each worker collected their urine void during a typical workweek involving spraying of folpet and harvest activities in a previously treated area.

[b] Each worker performed both spraying and harvest activities, which were separated by a minimum of two weeks.

[c] Total duration of spraying and harvest activities for each worker.

[d] Folpet were sprayed and harvest activities were performed only on workday 1, except for worker 2, who harvested from workdays 1 to 3.

Figure captions

Fig. 1 Chemical structure of (**a**) folpet and its main ring-metabolites: (**b**) phthalimide, (**c**) phthalamic acid and (**d**) phthalic acid.

Fig. 2 Representative chromatograms of TMS-PA and TMS-IS in blank human urine spiked with 12.1 µmol/L of PA and 6.72 µmol/L of *p*-methylhippuric acid (IS), along with the mass spectra and structure of both TMS derivatives.

Fig. 3 Representative chromatograms of TMS-PA and TMS-IS in human urine: (**a**) blank urine spiked with 12.1 µmol/L of PA and 6.72 µmol/L of methylhippuric acid (IS); (**b**) urine from a non-occupationally exposed person spiked with 6.72 µmol/L of IS; (**c**) urine sample of a volunteer orally dosed with folpet (9 h following absorption) spiked with 6.72 µmol/L of IS; (**d**) urine sample of a volunteer dermally dosed with folpet (24 h following application) spiked with 6.72 µmol/L of IS; (**e**) urine sample of a farmer spraying folpet (13 h following the beginning of spraying).

Fig. 4 Concentration-time profiles of total PA equivalents (mean ± SD) in the urine of volunteers administered folpet by the oral (1 mg/kg) (-●-) or dermal (10 mg/kg) (-○-) route.

Figure 1

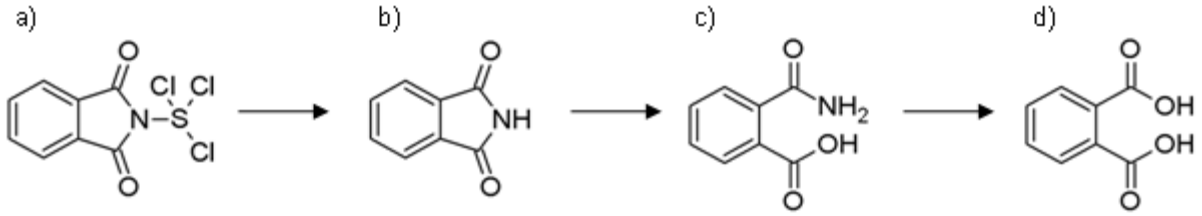
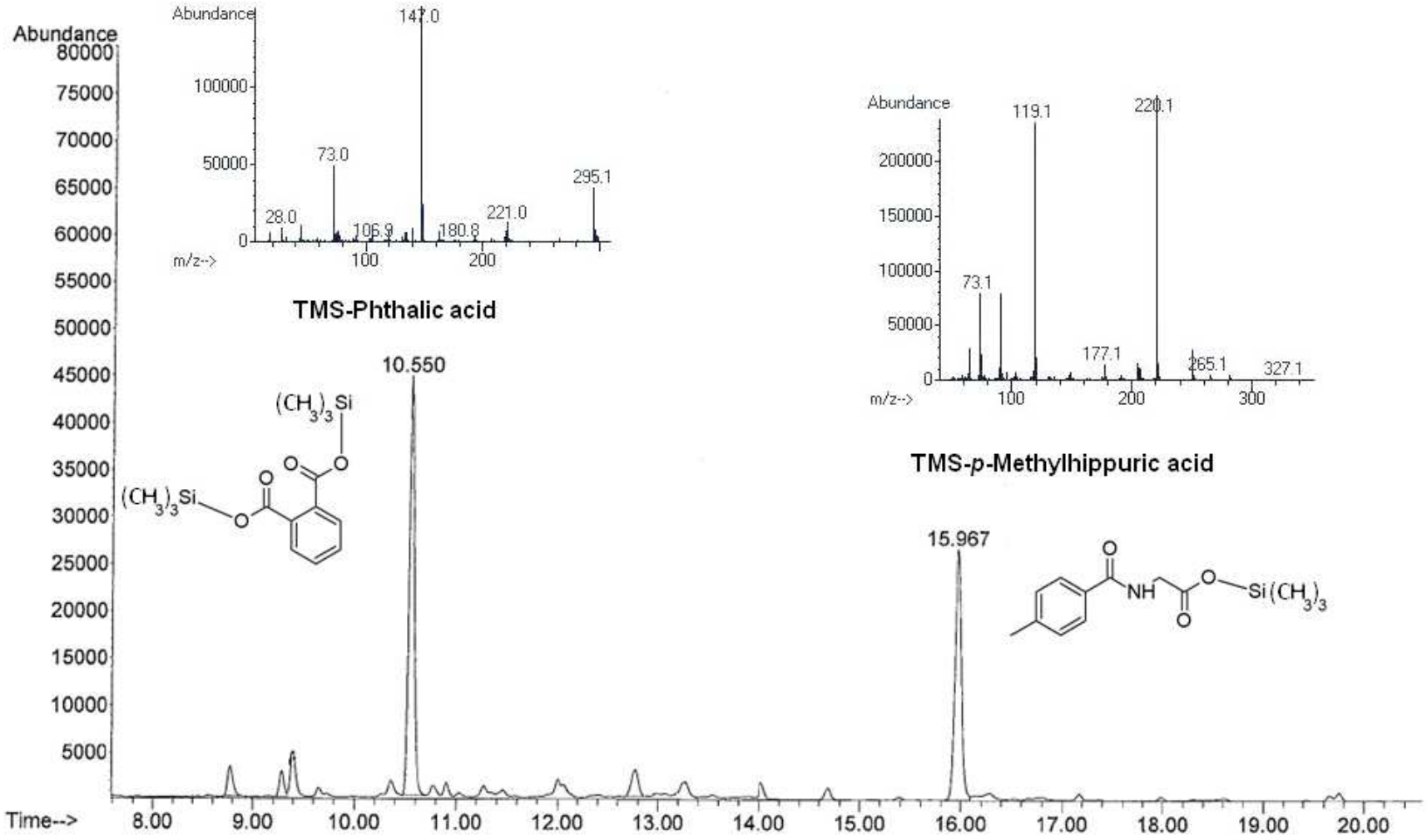


Figure 2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60000
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50000
45000
40000
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30000
25000
20000
15000
10000
5000
Time-->
8.00
9.00
10.00
11.00
12.00
13.00
14.00
15.00
16.00
17.00
18.00
19.00
20.00
10.550
15.967
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295.1
28.0
106.9
180.8
221.0
m/z-->
100
200
TMS-Phthalic acid
Abundance
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177.1
265.1
327.1
m/z-->
100
200
300
TMS-p-Methylhippuric acid

Figure 3

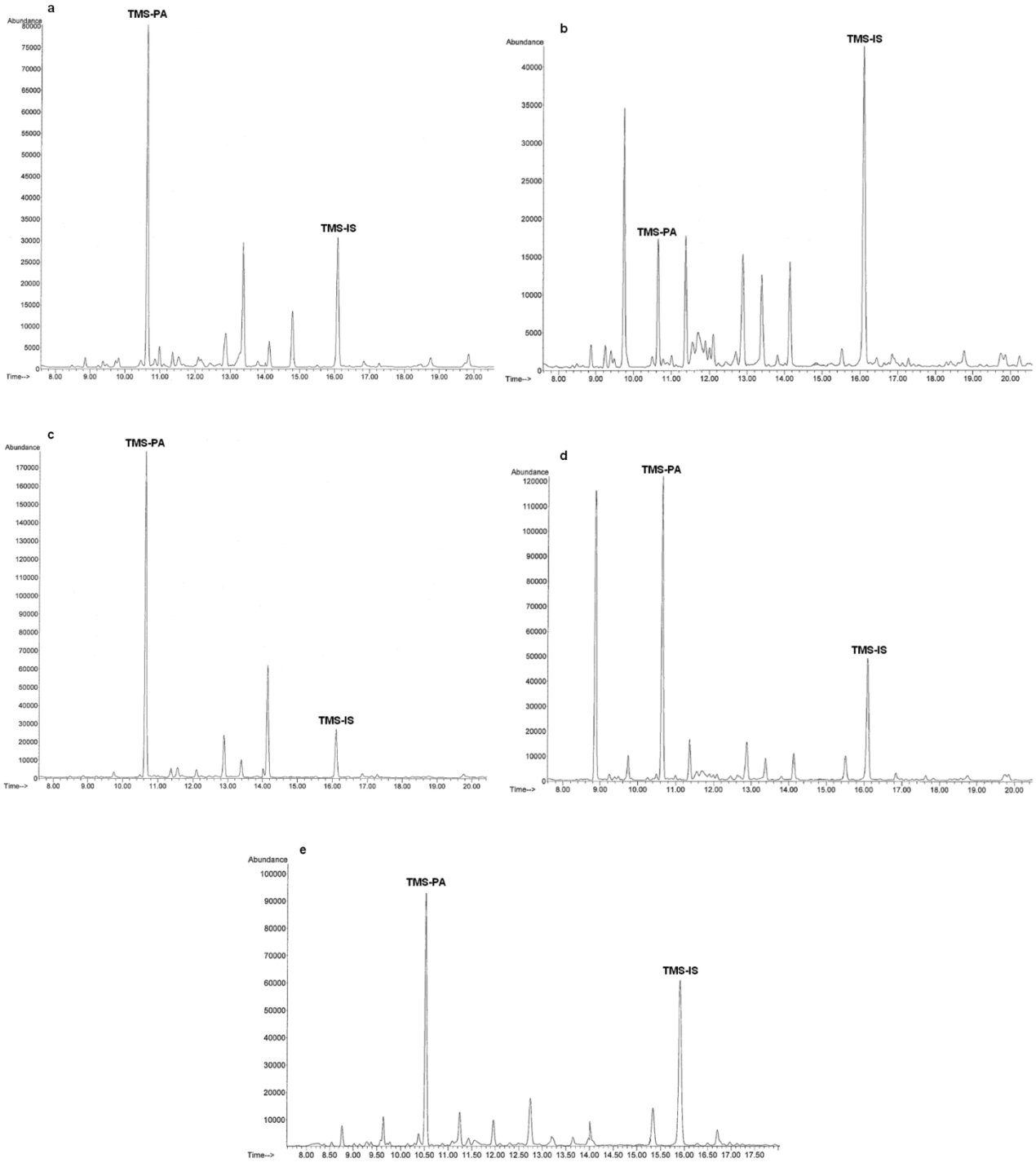
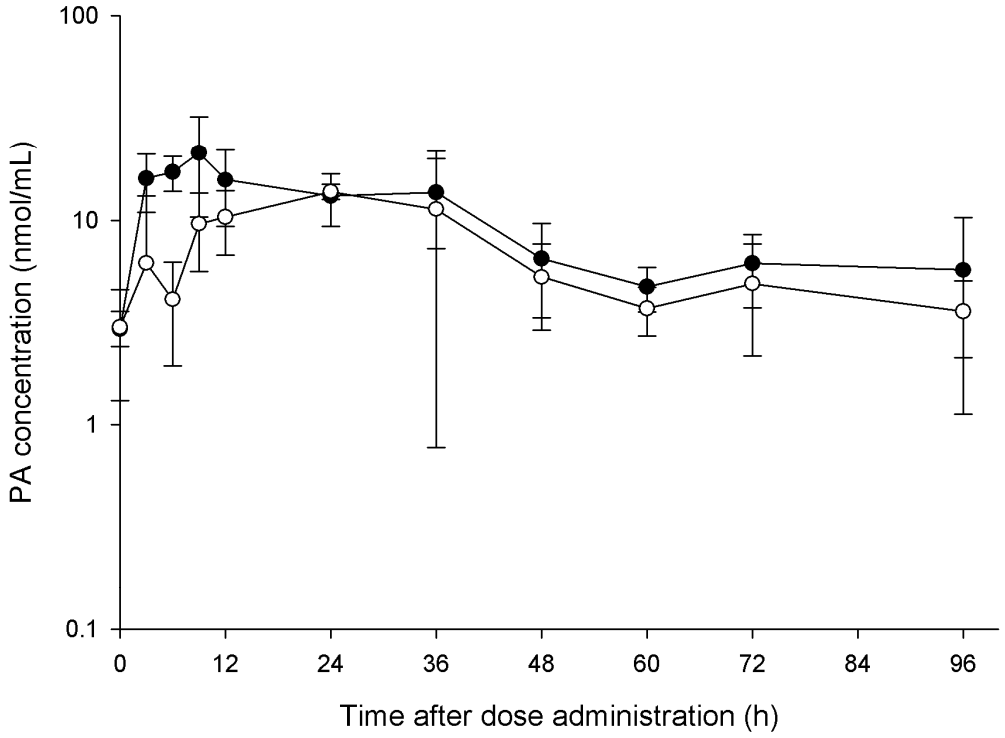

a
TMS-PA
TMS-IS
b
TMS-IS
TMS-PA
c
TMS-PA
TMS-IS
d
TMS-PA
TMS-IS
e
TMS-PA
TMS-IS

Figure 4


100
10
1
0.1
PA concentration (nmol/mL)
0
12
24
36
48
60
72
84
96
Time after dose administration (h)