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Quantification methods of folpet degradation products in plasma with HPLC-UV/DAD: Application to an *in vivo* toxicokinetic study in rats

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Abstract

Solid-phase extractions followed by HPLC-UV/DAD methods were developed for occupational biological monitoring or forensic investigations of the fungicide folpet using its degradation products, phthalimide and phthalamic acid as plasma biomarkers. These methods show good linearity ($r > 0.9955$), precision (CV < 15%) and accuracy (bias < 14.8%). The lower limits of quantification for phthalimide and phthalamic acid were 10 and 20 ng/ml and the absolute recoveries were higher than 86% and 68%, respectively. Applying these methods, a plasma toxicokinetic study of folpet in rats after intratracheal administration of Folpan 80WG® showed that inhalation of folpet could be a route of exposure with an important systemic absorption.

Keywords: Fungicide; Folpet; Phthalimide; Phthalamic acid; HPLC; Rat; Intratracheal toxicokinetic study

1. Introduction

Folpet, 2-[(trichloromethyl)thio]-1H-isoindole-1,3(2H)-dione (Fig. 1), is a contact fungicide belonging to the dicarboximide family with a mode of action centred around its reaction with thiol groups [1,2]. Used for the past 50 years, it is still widely employed on grapevines, fruit trees and vegetables as a preventive treatment against mildew, gray mold, spoilage fungi and wood rot fungi [3]. It is sprayed in high quantities (kg/ha) with a frequency of application around 10–14 treatments per year from May to September [4]. Folpet is classified as a harmful substance with possible risks of irreversible effects and to be noxious by inhalation [3,5]. Folpet and the other dicarboximide compounds have been found to be the pesticides both the most irritant for the skin, the most sensitizing [6,7]. Toxic effects have been reported both *in vitro* [8–11] and *in vivo* [12].

Being slightly water-soluble [1], folpet remains on the surface of treated plants in a particle form [8] and has been found to contaminate the environment, especially water [13,14] and air [4,15,16]. In French vine-growing regions, folpet has been found to be the main pesticide found in the air (rural and urban) both in terms of quantity and frequency [16–18]. Analytical methods using gas chromatography [14,19–24] or liquid chromatography [25–29] have been developed for folpet multiresidue analysis for regulatory monitoring of pesticide levels in food and water as well as for environmental detection.

People the most exposed to folpet particles are those who manipulate and spray the product in the course of their work. Dermal and inhalation exposure could represent possibly the greatest types of exposure for workers. Dermal deposition has been evaluated to be as great as 19 mg/h of work using patches placed on the skin. Inhalation exposure was recorded

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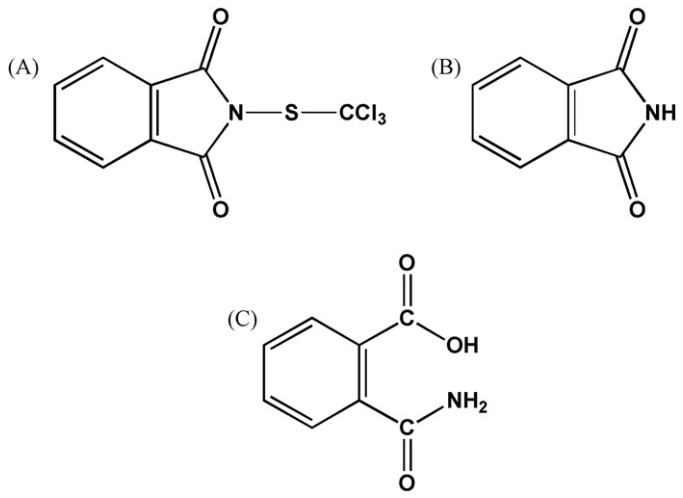

Fig. 1. Chemical structure of (A) folpet and its main degradation products, (B) phthalimide and (C) phthalamic acid.

to be up to 68 μg/h of work using personal air sampling pump (Panametrics®) (Baldi, unpublished data). Despite this, the internal dose received by workers exposed to folpet has not yet been published and no biological monitoring has been performed. In animal toxicokinetic studies, folpet has been reported to be absorbed rapidly into the plasma following a single oral dose [30] and to have low systemic absorption through the skin following dermal exposure [31]. These data showed that dermal exposure is not an important route of folpet systemic passage. On the other hand, little is known about its systemic absorption after inhalation. The degree to which these people are exposed should be assessed using biomarkers. To our knowledge, only one article has been published regarding human plasma levels of phthalimide, a degradation product of folpet [32].

Because of its short biological half-life (4.9 s) when exposed to blood *in vitro* [33], folpet could not directly be detected and assayed in plasma for biological monitoring. It is degraded either by hydrolysis or by reaction with thiol compounds [30,34,35] in to phthalimide and thiophosgene [36], the latter being a highly reactive and rapidly degraded compound [37]. Phthalimide is hydrolyzed to phthalamic acid (Fig. 1) and then to phtalic acid [36]. It has been previously reported that the principal degradation products of folpet both in an aqueous suspension [8] and administered per os were phthalimide and phthalamic acid, the latter representing approximately 80% of the original dose [30]. Phthalamic acid can further degrade to form phtalic acid, a multisource molecule frequently found in blood plasma. With this in mind and owing to a lack of data regarding the toxicokinetics of folpet following respiratory exposure, we analysed the two first degradation products of folpet (phthalimide and phthalamic acid) via intratracheal instillation. To our knowledge, only one method is available to assay phthalimide only in plasma [38]. No method is currently published for phthalamic acid.

The aim of this work was to develop and validate simple analytical methods for occupational biological monitoring, toxicokinetic and forensic investigations using phthalimide and phthalamic acid as the main plasma biomarkers of folpet exposure. A solid-phase extraction coupled with reversed phase HPLC-UV/DAD technique was employed. To assess the systemic absorption of folpet via the respiratory tract, these methods were then used in a toxicokinetic study in rats following an intratracheal administration of Folpan 80WG®, a commercial formulation of folpet.

2. Experimental

2.1. Reagents and chemicals

Analytical standards of phthalimide and phthalamic acid were purchased from Fluka (Saint Quentin-Fallavier, France) and Sigma–Aldrich (Saint Quentin-Fallavier, France), respectively. Paranitroaniline and nalidixic acid, the two internal standards (IS) were obtained from Sigma–Aldrich. The purity of these compounds was greater than 99% except phthalamic acid (purity > 97%). Folpan 80WG® was purchased from Euralis (Bruges, France). Orthophosphoric acid, potassium dihydrogen phosphate, boric acid and diphosphoric acid were from Merck (Fontenay Sous Bois, France). Propan-2-ol, acetonitrile, dichloromethane and formic acid were purchased from JT Baker (Deventer, Holland), methanol from Carlo Erba (Val de Reuil, France). All solvents were of HPLC-grade quality. Water was deionized and purified using a Milli-Q system (Millipore, Saint Quentin-en-Yvelines, France). Heparinized plasma of healthy human donors was purchased from the Etablissement de Transfusion Sanguine d'Aquitaine (ETSA, Bordeaux, France).

2.2. Stock and standard solutions

Stock solutions of phthalimide and phthalamic acid for generating calibration curves, were prepared at a concentration of 1 mg/ml in acetonitrile and methanol, respectively. They were stored at −20 °C and stable for at least 4 months. The 1 mg/ml paranitroaniline stock solution in methanol was diluted daily in 10 mM KH_2PO_4 solution (pH 3.4, adjusted with 0.01 M orthophosphoric acid) to yield a 1 μg/ml working solution. The 1 mg/ml nalidixic acid stock solution in acetonitrile was diluted daily in 0.1 M boric acid/0.1 M diphosphoric acid mixture (50/50; v/v) to yield a 200 ng/ml working solution.

The standard solutions for calibration curves were prepared by serial dilution of stock solutions in drug-free plasma to give final concentrations of 10, 20, 40, 60 and 100 ng/ml for phthalimide and 20, 50, 100, 150 and 200 ng/ml for phthalamic acid. Linear calibration curves were obtained by plotting the compound-to-internal standard peak area ratios versus the concentrations in ng/ml. For the preparation of quality controls, independent stock solutions were prepared and further diluted in drug-free plasma to obtain concentrations of 10, 15, 30 and 80 ng/ml for phthalimide and 20, 60 and 120 ng/ml for phthalamic acid.

2.3. Equipment and chromatographic conditions

The high-performance liquid chromatography system was a Hewlett-Packard (HP) 1100 model with a quaternary pump, an automatic injector and a diode array ultra-violet detec-

Table 1
Mobile phase gradient HPLC programs used for phthalimide and phthalamic acid assay

Time (min)	10 mM KH_2PO_4 solution[a] (%)	Acetonitrile (%)
Phthalimide method		
0	70	30
0.5	70	30
4	60	40
5.3	60	40
9	20	80
9.3	20	80
12.3	70	30
15	70	30
Phthalamic acid method		
0	92.5	07.5
4	92.5	07.5
12	30	70
13	30	70
16	92.5	07.5
19	92.5	07.5

[a] pH 3.4, adjusted with 0.01 M H_3PO_4.

tor coupled with an HP ChemStation 6.0 system (Interchim, Montluçon, France). The chromatographic separation was performed at 25 °C, on a dC18 Atlantis® reversed-phase column (150 × 4.6 mm, 5 μm, Waters, St Quentin-en-Yvelines, France). The mobile phase consisted of a gradient of acetonitrile and 10 mM KH_2PO_4 solution (pH 3.4, adjusted with 0.01 M orthophosphoric acid) (Table 1) and was delivered at 1 ml/min flow rate. Before use, the mobile phase was filtered using a 0.2 μm nylon membrane. The compounds were detected at 218 nm wavelength for phthalimide, 380 nm for paranitroaniline, 200 nm for phthalamic acid and 320 nm for nalidixic acid.

2.4. Extraction procedure

2.4.1. Phthalimide extraction method

The phthalimide was extracted using solid-phase extraction cartridges (Oasis® 1 ml, 10 mg, Waters) that contain HLB (Hydrophilic Lipophilic Balance) sorbent. Each cartridge was equilibrated by 1 ml of dichloromethane followed by 1 ml of methanol and then conditioned by 1 ml of water. One millilitre of plasma with 1 ml of water was vortexed, loaded onto and passed through the cartridge. After one wash with 1 ml of 0.1 M H_3PO_4 and once with 1 ml of 0.1 M H_3PO_4/methanol mixture (80/20; v/v), phthalimide was then eluted using 1 ml of dichloromethane. The eluent was evaporated at ambient temperature using a Speed Vac plus SC 110A (Savant, Fisher Scientific Bioblock, Illkirch, France). The pellet was resuspended in 150 μl of 10 mM KH_2PO_4 aqueous solution (pH 3.4, adjusted with H_3PO_4) containing 1 μg/ml paranitroaniline. After vortexing, the resulting solution was then transferred to an injection vial from which a sample of 100 μl was then injected onto the HPLC column.

2.4.2. Phthalamic acid extraction method

The phthalamic acid was extracted using solid-phase extraction cartridges (Oasis® 1 ml, 30 mg, Waters) that contain HLB (Hydrophilic Lipophilic Balance) sorbent. Each cartridge was equilibrated by 1 ml of dichloromethane followed by 1 ml of methanol and then conditioned by 1 ml of 0.01 M H_3PO_4. Five hundred microlitres of plasma with 500 μl of 0.1 M boric acid/0.1 M diphosphoric acid mixture (50/50; v/v) containing 200 ng/ml nalidixic acid as internal standard was vortexed, loaded onto and passed through the cartridge. After one wash with 1 ml of 0.01 M H_3PO_4, phthalamic acid was then eluted using 1 ml of dichloromethane/propan-2ol/formic acid mixture (89.8/10/0.2; v/v/v). The eluent was evaporated at ambient temperature using a Speed Vac plus SC 110A. The pellet was resuspended in 150 μl of distilled water. After vortexing, the resulting solution was then transferred to an injection vial from which a sample of 100 μl was then injected onto the HPLC column.

2.5. Validation procedure

The analytical methods were validated on human heparinized blank plasma spiked with phthalimide and phthalamic acid. Linearity was assessed by linear regression from four independent replicates of five standard solutions of the calibration range. Method validation was performed by extracting four replicates of spiked plasma samples at 10, 15, 30 and 80 ng/ml of phthalimide and at 20, 60 and 120 ng/ml of phthalamic acid during the same working day (intra-day variation) and on four different days (inter-day variation). The coefficient of variation (CV) and bias were calculated for determination of precision and accuracy, respectively. The limit of quantification was defined as the lowest concentration where data could be obtained with precision and accuracy within ±20% of the nominal value. Absolute recovery was determined by checking spiked plasma samples at known concentrations of 10, 30, 80 ng/ml for phthalimide and 20, 60, 120 ng/ml for phthalamic acid. Four replicates were used for each concentration. The short-term stability of the compounds was studied in plasma samples after storage at +4 °C for 24 h. The long-term stability was assessed after storage at −20 °C for 30 days. Three different control levels were tested for each compound ($n = 4$ replicates by control level). The post-preparative stability was conducted by analyzing extracted plasma samples kept under autosampler conditions (+20 °C) for 24 h.

2.6. Application

Plasma toxicokinetic study in rats after intraperitoneal or intratracheal administration of Folpan 80WG®, a commercial formulation of folpet.

2.6.1. Animals

Principles of laboratory animal care (NIH Publication No. 85-23 revised 1985) were followed. Male Wistar rats with a body weight of approximately 400 g were purchased from R. Janvier (Le Genest-Saint-Isle, France). Animals were housed in a temperature-controlled environment (22 ± 1 °C) with a 12 h light–dark cycle and daily feedings of standard chow pellets (UAR, France) and water ad libitum. They were acclimatized to this environment for at least 3 days before experimentation.

2.6.2. Experimental design

The dose of folpet was prepared using Folpan 80WG®, a commercial formulation containing 80% (w/w) folpet as the active ingredient and was suspended at 10 mg/ml (folpet) in sterile 0.9% NaCl which was vortexed for 1 min before administration.

The animals were given a single dose of folpet (10 mg/kg) by intraperitoneal injection or intratracheal instillation. Prior to intratracheal dosing, animals were anesthetized by an intraperitoneal injection of a mixture of 50 mg/kg ketamine and 4 mg/kg xylazine by body weight. Rats were held in a supine position whilst, the neck area was illuminated by a lamp. The intratracheal instillation was conducted using an 18 Gauge cannula inserted via the mouth into the trachea. The Folpan 80WG® suspension was instilled at a volume of 1 ml/kg via a sterile 1 ml syringe and followed by insufflation with 0.3 ml of air.

Rats were sacrificed after 0, 0.25, 0.5, 1, 2, 4, 8 and 24 h, following a single intraperitoneal or intratracheal administration, four to five rats per time point. Animals were anesthetized by intraperitoneal injection of thiopental sodium (50 mg/kg). The thorax was opened, blood was collected via heart puncture using a heparinized syringe. The blood was centrifuged for 10 min at 2500 × *g*. The plasma was collected, aliquoted and stored at −20 °C until analysis. Phthalimide and phthalamic acid concentrations in each sample were determined by the HPLC-UV/DAD method described above, using blank male rat plasma to establish the calibration curve.

2.6.3. Calculations and toxicokinetic analysis

All rat plasma concentrations were expressed as mean followed by standard error (mean ± S.E.). Toxicokinetic analysis was performed on the plasma concentration–time data using R2.4.1 software (Free Software Foundation, Boston, USA). The area under the curve from time zero to last time of sampling ($AUC_{0\rightarrow t}$) was calculated by the trapezoidal rule. The AUC from the last experimental time to infinity ($AUC_{t\rightarrow\infty}$) was calculated by extrapolation, dividing the last measured plasma concentration value by the terminal elimination rate constant (β) corresponding to calculated from the terminal linear portion of the plasma log concentration–time curve. The $AUC_{0\rightarrow\infty}$ was calculated as the sum of $AUC_{0\rightarrow t}$ and $AUC_{t\rightarrow\infty}$. The terminal elimination half-life ($t_{1/2}$) was calculated as $t_{1/2} = \ln 2/\beta$. Graphical analyses were carried out using Microsoft Excel (Microsoft Corp., Redmond, WA, USA). C_{max} and T_{max} values were obtained directly from the observed concentration versus time data.

3. Results and discussion

The methods developed here used an HPLC system coupled with an UV-diode array detector. Several HPLC columns were tested and a dC18 Atlantis® reversed-phase column was chosen as it allowed retention of both phthalamic acid and phthalimide. For phthalamic acid retention, a polar and acidic mobile phase was necessary, starting with 92.5% of 10 mM KH_2PO_4 solution (pH 3.4 adjusted H_3PO_4).

A solid-phase extraction procedure was developed for elimination of plasma endogenous interferences, sample purification and compound concentration. The advantage of using solid-phase extraction compared to liquid-liquid extraction is that it requires less time and solvent, it is simple, efficient and can be automated. Different solvents for compound elution from the HLB oasis® sorbent cartridge were tested. Dichloromethane was found to be the best eluting solvent among those tested (ether, methanol, chloroform) because of a good recovery with a good baseline and less plasma endogenous interferences on the chromatogram. It was necessary to use two different solid-phase extraction protocols and analytical methods because of the difference in the polarity between phthalimide and phthalamic acid. Moreover, the stability of both compounds in an aqueous solution was pH-dependent with an incompatibility of the solution used to resuspend the pellet after extraction: phthalimide being stable in acidic conditions (pH 3.4) and phthalamic acid being stable in pH-neutral conditions.

The recovery of phthalamic acid required plasma acidification before loading onto higher capacity cartridges (30 mg HLB Oasis®) and also required formic acid in the eluting solution. Propan-2-ol was added in the eluting solution for a better mix between dichloromethane and formic acid.

3.1. Chromatography

Representative chromatograms of a blank plasma (Fig. 2a and c) and plasma spiked with 10 ng/ml of phthalimide (Fig. 2b) and with 20 ng/ml of phthalamic acid (Fig. 2d) following solid-phase extraction and HPLC-UV/DAD analysis are presented in Fig. 2. The chromatograms of blank plasma show no interference from endogenous plasma substances, indicating the efficiency of the clean up method used and proving the selectivity of the method. Purity of the peak was analyzed using molecular spectra obtained with the UV-diode array detector. A very good repeatability of the retention time for all compounds was achieved: 4.67 min for phthalimide, 7.18 min for paranitroaniline, 5.32 min for phthalamic acid and 13.03 min for nalidixic acid. The total run time was 15 min for phthalimide assay and 19 min for phthalamic acid assay.

3.2. Linearity

Linearity of standard calibration curves in these methods was obtained over a range between 10 and 100 ng/ml for phthalimide ($n = 4$) and 20–200 ng/ml for phthalamic acid ($n = 4$). The methods revealed good linearity for the two compounds with a correlation coefficient of 0.9981 for phthalimide ($y = 0.0278x + 0.0843$) and 0.9955 for phthalamic acid ($y = 0.0152x + 0.0097$).

The calibration was also linear up to 500 ng/ml for phthalimide and up to 5000 ng/ml for phthalamic acid. This was evaluated during the toxicokinetic study in rat plasma.

3.3. Precision, accuracy and sensitivity

These methods were accurate and precise for the two compounds. Results obtained for precision and accuracy are listed

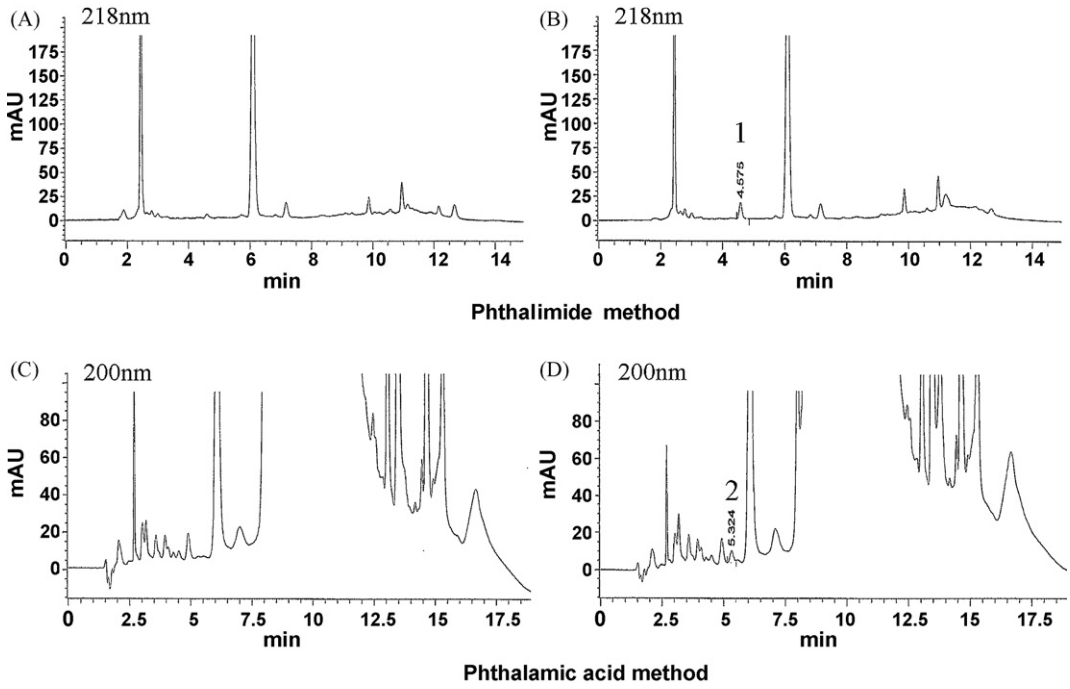

Fig. 2. Representative chromatograms using the phthalimide HPLC-UV/DAD method (218 nm): (A) blank plasma and (B) plasma spiked with 10 ng/ml of (1) phthalimide. Representative chromatograms using the phthalamic acid HPLC-UV/DAD method (200 nm): (C) blank plasma and (D) plasma spiked with 20 ng/ml of (2) phthalamic acid.

Table 2
Data of assay validation obtained for each day in spiked plasma for phthalimide and phthalamic acid

Concentration (ng/ml)	Day 1		Day 2		Day 3		Day 4	
	Mean ± S.D. (ng/ml, $n=4$)	Bias (%)	Mean ± S.D. (ng/ml, $n=4$)	Bias (%)	Mean ± S.D. (ng/ml, $n=4$)	Bias (%)	Mean ± S.D. (ng/ml, $n=4$)	Bias (%)
Phthalimide								
10	8.3 ± 0.9	−17.0	8.3 ± 0.7	−17.0	8.3 ± 0.3	−17.0	8.0 ± 0.2	−20.0
15	13.6 ± 0.5	−9.3	13.8 ± 2.1	−8.0	12.9 ± 0.4	−14.0	15.7 ± 0.5	+4.6
30	31.8 ± 0.3	+6.0	29.4 ± 1.1	−2.0	28.9 ± 2.2	−3.7	28.7 ± 2.5	−4.3
80	80.6 ± 1.8	+0.8	80.6 ± 3.0	+ 0.8	74.1 ± 0.9	−7.4	79.8 ± 1.9	−0.3
Phthalamic acid								
20	20.4 ± 0.3	+2.0	22.9 ± 1.2	+14.5	21.6 ± 1.4	+8.0	20.2 ± 1.4	+1.0
60	51.1 ± 7.3	−14.8	61.2 ± 7.1	+2.0	62.0 ± 1.2	+3.3	55.2 ± 3.5	−8.0
120	127.1 ± 9.8	+5.9	119.4 ± 8.3	−0.5	117.8 ± 7.9	−1.8	113.4 ± 6.5	−5.5

in Tables 2 and 3. A good inter and intra-assay accuracy and a good inter and intra-assay precision were achieved with a bias and a coefficient of variation (CV) less than 15% for the two compounds. The limit of quantification (LOQ) was 10 ng/ml for phthalimide and 20 ng/ml for phthalamic acid with a good inter and intra-assay accuracy and precision (bias and CV < 20%). The limit of detection (LOD) was 5 ng/ml for phthalimide and 10 ng/ml for phthalamic acid.

3.4. Absolute recovery

The absolute recoveries of phthalimide and phthalamic acid for each control level are presented in Table 4. The mean recoveries of phthalimide and phthalamic acid were 90% ± 6.9 and 76% ± 9.4 (mean ± S.D., $n=12$), respectively.

Table 3
Precision and accuracy of the determination of phthalimide and phthalamic acid in spiked plasma

Concentration (ng/ml)	Intra-assay precision (CV, %, $n=4$, day no. 3)	Inter-assay precision (CV, %, $n=16$)	Bias (%)
Phthalimide			
10	3.7	6.7	−18.2
15	3.1	11.8	−7.4
30	7.6	7.4	−2.5
80	1.2	5.3	−2.8
Phthalamic acid			
20	6.5	7.1	+5.9
60	1.9	9.2	−2.6
120	6.7	6.3	−2.6

CV: coefficient of variation.

Table 4
Phthalimide and phthalamic acid absolute recovery for each plasma concentration

Concentration (ng/ml)	Absolute recovery (%) (mean ± S.D., $n=4$)
Phthalimide	
10	99 ± 1.9
30	87 ± 4.2
80	86 ± 2.6
Phthalamic acid	
20	68 ± 2.1
60	72 ± 4.7
120	87 ± 6.2

Table 5
Stability of phthalimide and phthalamic acid in plasma after 30 days of storage at −20 °C ($n=4$)

Concentration (ng/ml)	Mean ± S.D. (ng/ml)	CV (%)	Bias (%)
Phthalimide			
10	8.02 ± 0.19	2.39	−19.85
30	25.04 ± 1.26	5.04	−16.53
80	72.02 ± 2.95	4.09	−9.97
Phthalamic acid			
20	19.46 ± 1.15	5.89	−2.70
60	65.07 ± 5.40	8.30	+8.46
120	127.06 ± 9.78	7.70	+5.88

3.5. Stability

During the development of the methods, it was noted that phthalimide was unstable in plasma at +20 °C over 24 h (bias = 30%). A short-term stability study was conducted for phthalimide with plasma samples kept at +4 °C for 24 h. No degradation was found (bias = −16% for 10 ng/ml, −8.7% for 30 ng/ml and −7.7% for 80 ng/ml). A long-term stability study was performed by storage at −20 °C over 30 days (Table 5). Phthalimide degradation in frozen plasma was not very important (losses of 10–15.5%). For the LOQ level of phthalimide, bias value at day 30 was similar to bias values obtained from freshly prepared plasma (Tables 2 and 3). Moreover, a bias value below 20% for the LOQ is correct for an analytical method. Phthalamic acid was relatively stable at −20 °C with an increase around 5–8% corresponding possibly, at least in part, to the hydrolysis of phthalimide. In light of these results, it should be recommended that plasma samples be kept at +4 °C after collection and be assayed in the first 24 h or these should be stored at −20 °C until analysis.

The post-preparative stability of the compounds was studied after the solid-phase extraction under autosampler conditions (+20 °C) for 24 h. Phthalimide was stable with a bias value below 10%. Owing to trace formic acid persisting after the evaporation step, phthalamic acid was not stable after 6 h storage in the autosampler (bias = 27%). For phthalamic acid, if several samples should be injected the same day, it is recommended after the evaporation step to keep extracted samples at −20 °C before injection into the HPLC system.

3.6. Application: Plasma toxicokinetic study after intraperitoneal or intratracheal administration of Folpan 80WG®, a commercial formulation of folpet in rat

People, who manipulate folpet could be exposed by the respiratory route. However, systemic absorption of this pesticide through this route of exposure has not yet been reported. Applying the analytical methods validated in this work, a toxicokinetic study of folpet in rats after intratracheal or intraperitoneal Folpan 80WG® administration was performed. Because excipients can influence the toxicokinetic of a compound, particularly its absorption, a commercial form of folpet (Folpan 80WG®) was chosen, containing only folpet as active ingredient. Previous folpet toxicokinetic studies have employed technical-grade folpet; the use here of a commercial form is more close to the real-life conditions of exposure. In usual conditions of use, commercial forms of folpet have been found to be present as a particle form with a size small enough to be inhaled by humans [8].

Intratracheal administration, an experimental route widely use for inhalation exposure of particles in animals [39] was chosen to assess the toxicokinetic of folpet after respiratory exposure. The intraperitoneal route was used as the reference model because particles of folpet are not directly administrable in the bloodstream.

After Folpan 80WG® administration, rats were sacrificed at different times, plasma samples were collected, extracted and analyzed as described earlier. Standard calibration curves were prepared in blank rat plasma and extended until 500 ng/ml for phthalimide and until 5000 ng/ml for phthalamic acid. Good linearity was achieved ($r=0.9972$ for phthalimide, $r=0.9975$ for phthalamic acid, $n=4$). Blank rat plasmas were tested with these analytical methods; no endogenous interference was detected and the same sensitivity and recovery were noted compared to human plasma (data not shown).

In rat plasma, both folpet degradation products, phthalimide and phthalamic acid were identified and quantified for both intraperitoneal and intratracheal administration. These data show that folpet is released from the particles of Folpan 80WG® administered for both routes of administration and then, itself or its degradation products were absorbed. Folpet having a very short half-life in blood (4.9 s) and degraded on contact with thiol-compounds, it was not possible to quantify this compound in plasma and thus to specify whether folpet itself or its degradation products were absorbed.

Fig. 3 shows the mean plasma concentration–time profiles of phthalimide and phthalamic acid for the two routes of administration. The time to reach the maximum plasma concentration (T_{max}), the maximum plasma concentration (C_{max}), the area under the curve ($AUC_{0\rightarrow\infty}$) and the terminal half-life of elimination ($t_{1/2}$) estimated for each compound are summarized in Table 6.

For both routes of administration, the plasma concentrations of both folpet degradation products tested here increased rapidly and had comparable T_{max} values (around 0.25 h). This confirmed that folpet was rapidly degraded *in vivo* and showed that after intratracheal instillation in rats, most of

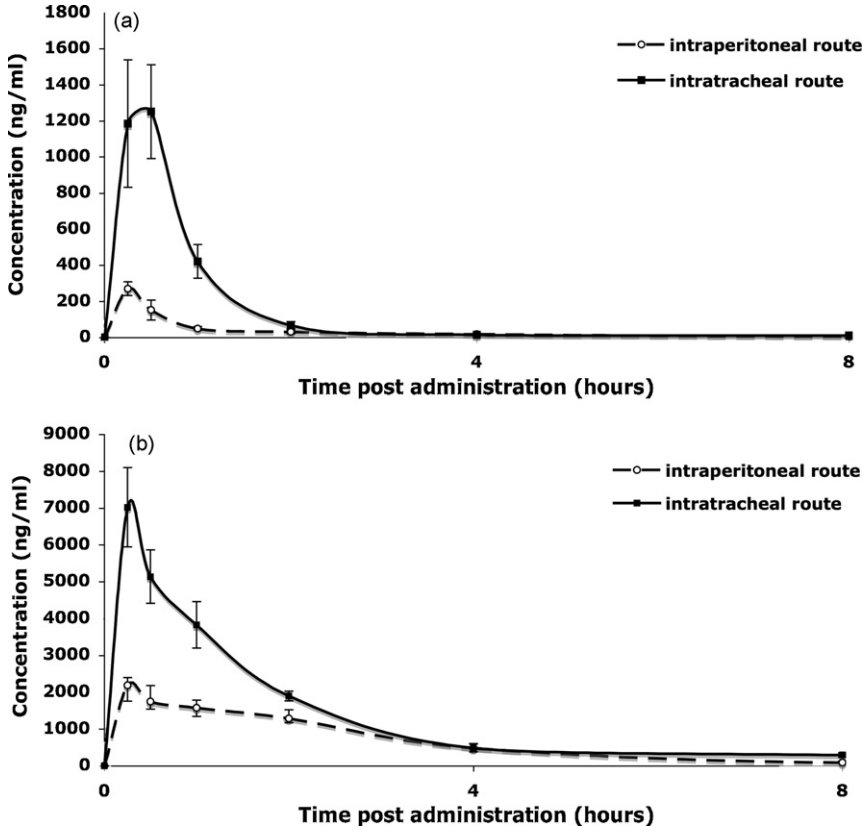

Fig. 3. Plasma concentration–time profile of phthalimide (a) and phthalamic acid (b). (a) Plasma concentration–time profile of phthalimide (mean ± S.E.) is represented, after a 10 mg/kg single dose of folpet by two different administration route (intratracheal or intraperitoneal) in male Wistar rats (n = 4–5 animals per time point). (b) Plasma concentration–time profile of phthalamic acid (mean ± S.E.) is represented, after a 10 mg/kg single dose of folpet by two different administration route (intratracheal or intraperitoneal) in male Wistar rats (n = 4–5 animals per time point).

the phthalimide was rapidly hydrolysed (<0.25 h) into phthalamic acid. Both phthalimide and phthalamic acid plasma concentrations declined rapidly in an apparently multiphasic manner due to several phenomena involved such as hydrolysis degradation, tissue distribution and renal elimination. For both routes of administration, the terminal elimination half-life was around 2.2–2.6 h for phthalimide and around 4.7–5 h for phthalamic acid. These rapid elimination half-lives demonstrate that these two degradation products did not accumulate in rats. Twenty-four hours following Folpan 80WG® administration, no phthalimide was detected in plasma samples (<5 ng/ml) and phthalamic acid was close to the lower limit of quantification (~25 ng/ml). This *in vivo* profile of the two main degradation products of folpet was in close agreement with previously reported values for folpet toxicokinetics in rats following oral [30] or intraperitoneal administration [34].

The two compounds were found in higher quantity following Folpan 80WG® intratracheal instillation than intraperitoneal administration with a C_{max} 4.6 and 3.2-fold higher and an $AUC_{0\rightarrow\infty}$ 3.2 and 2.0-fold higher for phthalimide and phthalamic acid, respectively. The difference in $AUC_{0\rightarrow\infty}$ values between intratracheal and intraperitoneal routes could possibly be explained by the fact that in the respiratory tract there is a large area of exchange between air and blood with a high blood flow. These characteristics could favour a better release of folpet from Folpan 80WG® particles, and a more complete dissolution of folpet than in the peritoneum.

Table 6
Toxicokinetic parameters obtained after a single dose of 10 mg/kg folpet in rats

Route	Phthalimide		Phthalamic acid	
	Intraperitoneal	Intratracheal	Intraperitoneal	Intratracheal
T_{max} (h)	0.25	0.25–0.5	0.25	0.25
C_{max} (μg/l), mean ± S.E.	270.63 ± 38.02	1252.10 ± 260.05	2186.78 ± 206.47	7020.22 ± 2155.67
$t_{1/2}$ (h)	2.62	2.19	4.97	4.68
$AUC_{0\rightarrow\infty}$ (μg h/l)	461.61	1469.11	7196.43	14209.45

T_{max}: time to reach the maximum plasma concentration; C_{max}: maximum plasma concentration; $t_{1/2}$: terminal half-life of elimination; $AUC_{0\rightarrow\infty}$: area under the curve from time zero to infinity.

Phthalimide had a terminal elimination half-life half as long as that of phthalamic acid. This was probably due, at least in part, to its degradation into phthalamic acid. The latter was the main degradation product for both administration routes. Indeed, after intratracheal administration, the C_{max} of phthalamic acid was 5.6-fold higher than the C_{max} of phthalimide and the $AUC_{0\rightarrow\infty}$ was 9.7-fold higher. This toxicokinetic study allows us to suggest phthalamic acid as a biomarker of exposure to folpet both due to its better persistence and greater plasma concentration compared to phthalimide. Moreover, this study shows the applicability of the analytical methods developed here. The important systemic absorption following intratracheal administration of folpet in a particle form reported here shows that the respiratory exposure should not be neglected in the working environment.

4. Conclusion

The methods described here for the quantification of phthalimide and phthalamic acid in plasma are reliable and simple. The solid-phase extraction followed by reversed high-performance liquid chromatographic methods coupled with an ultra-violet-diode array detector allow good recovery, sensitivity, linearity, accuracy and precision.

The plasma toxicokinetic study of folpet in rats after intratracheal administration of Folpan 80WG® suggests that inhalation of folpet particles could be a route of exposure with an important systemic absorption. Moreover, the results demonstrate that folpet degradation products could be employed as plasma biomarkers, particularly phthalamic acid. These methods could be applied for toxicokinetic or forensic investigations and could be useful for biological monitoring of workers exposed to folpet.

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