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Liquid chromatography - tandem mass spectrometry (LC/APCI-MS/MS) methods for the quantification of captan and folpet phthalimide metabolites in human plasma and urine

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Abstract

Captan and folpet are fungicides largely used in agriculture. They have similar chemical structures, except that folpet has an aromatic ring unlike captan. Their half-lives in blood are very short, given that they are readily broken down to tetrahydrophthalimide (THPI) and phthalimide (PI), respectively. Few authors measured these biomarkers in plasma or urine and analysis was conducted either by gas chromatography coupled to mass-spectrometry (GC-MS) or liquid chromatography with UV detection (LC-UV). The objective of this study was thus to develop simple, sensitive and specific liquid chromatography - atmospheric pressure chemical ionization-tandem mass spectrometry (LC/APCI-MS/MS) methods to quantify both THPI and PI in human plasma and urine. Briefly, deuterated THPI was added as an internal standard and purification was performed by solid phase extraction followed by LC/APCI-MS/MS analysis in negative ion mode for both compounds. Validation of the methods was conducted using spiked blank plasma and urine samples at concentrations ranging from 1 to 250 µg/L and 1 to 50 µg/L, respectively, along with samples of volunteers exposed to captan or folpet. The methods showed a good linearity ($R^2 > 0.99$), recovery (on average 90% for THPI and 75% for PI), intra- and inter-day precision (RSD < 15%) and accuracy (< 20%), and stability. The limit of detection was 0.58 µg/L in urine and 1.47 µg/L in plasma for THPI, and 1.14 and 2.17 µg/L, respectively, for PI. The described methods proved to be accurate and suitable to determine the toxicokinetics of both metabolites in human plasma and urine.

Keywords Tetrahydrophthalimide · Phthalimide · LC/APCI-MS/MS · Plasma · Urine

Introduction

Many winegrowers and tree farmers apply captan (1,2,3,6-tetrahydro-*N*-(trichloromethyl thio) phthalimide) or folpet (*N*-(trichloromethyl thio) phthalimide) to treat fungal diseases. These two common fungicides belong to the family of thiophthalimide pesticides. They have a very similar chemical structure, except that folpet has an aromatic ring while captan has a cyclohexene ring. Both compounds have very short half-lives in biological matrices, as assessed by Gordon *et al.* [1;2]. Captan was found to degrade with a half-life of 0.97 seconds in blood and folpet with a half-life of 4.9 seconds when ^{14}C-captan was directly added at 33.2 nmol/L and ^{14}C-folpet at 33.7 nmol/L to 1 mL of human blood [1]. Therefore, they are almost instantaneously metabolized to tetrahydrophtalimide (THPI) for captan and to phthalimide (PI) for folpet (Fig. 1). Unlike the two parent compounds, THPI and PI metabolites can be quantified in human plasma and urine [3-12]. Other metabolites of captan and folpet have also been identified in animal metabolism studies, namely 2-thiothiazolidine-4-carboxyl acid (TTCA) as a trichloromethyl thio derivative metabolite of captan, THPI derivatives, and phthalamic and phthalic acids as derivatives of PI [2;13-16]; nonetheless, THPI is the metabolite of captan most measured in the published human biomonitoring studies [5-12] while human biomonitoring data on the metabolites of folpet are limited [3;17].

The published analytical methods for the measurement of THPI and PI in human plasma or urine used either gas chromatography with mass spectrometry detection (GC-MS) [3;6;7;10] or liquid chromatography with UV detection (LC-UV) [4]. Liquid chromatography – mass spectrometry (LC-MS) methods with atmospheric pressure chemical ionization (APCI) have yet to be developed for these biomarkers. LC-APCI-MS analysis is known to be very specific to one

analyte and to improve sensitivity, especially for the detection of more polar or low-concentration compounds [18]. The objective of this work was thus to develop such analytical methods for the quantification of THPI and PI in human plasma and urine to determine the toxicokinetics of these biomarkers in volunteers as well as assess worker exposure to captan and folpet through biomonitoring.

Materials and methods

Chemicals and reagents

Reference standards of cis-1,2,3,6-tetrahydrophthalimide (THPI) and phthalimide (PI) (>99% purity) were obtained from Sigma-Aldrich (Buchs, St Gallen, Switzerland), while deuterated cis-1,2,3,6-tetrahydrophthalimide (THPI-d) (99% purity) was purchased from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). HPLC grade acetonitrile, methanol and dichloromethane were also obtained from Sigma-Aldrich (Buchs, St Gallen, Switzerland). Ammonium sulphate was purchased from Merck (Zug, Switzerland). Water was purified using a TKA GenPure water treatment system obtained from TKA Wasseraufbereitungsszsteme GmbH (Niederelbert, Germany).

Standard preparation

Stock standard solutions

Individual stock standard solutions were prepared by dissolving 100 mg of THPI or PI in 100 mL of methanol and 20 mg of THPI-d in 100 mL of methanol. They were stored at -20° C in a glass container until used.

Calibration curves

To prepare the calibration curves, stock standard solutions were diluted to obtain working solutions of THPI and PI standards at 10, 100, and 1 000 mg/L. These working standard solutions were kept at 4°C and were used daily to prepare calibration curves in urine and plasma, hence to spike blank urine from nonexposed persons at six concentration levels and blank plasma from

nonexposed persons at six concentration levels. The concentration levels were the same for both THPI and PI, thus 0.5, 1, 2, 5, 10 and 50 µg/L in urine and 1, 5, 10, 50, 100 and 250 µg/L in plasma.

Similarly, the stock internal standard solution was diluted to obtain a working solution at 250 µg/L. A constant volume of 125 µL of this solution was added to the calibration curve samples, which translates into a concentration of 62.5 µg/L in the 500 µL urinary and plasma extracts after sample processing.

Simultaneously, calibration points were also prepared in methanol from the same working solutions of THPI, PI and THPI-d standards. The six concentration levels were 1, 2, 5, 10, 15 and 20 µg/L for THPI and PI. Since final volume of each level was 1 ml, 250 µL of THPI-d working solution at 250 µg/L were thus added in this case, which corresponds to a concentration of 62.5 µg/L.

Sample treatment

Analysis of THPI and PI in urine

THPI and PI in urine were analyzed using liquid chromatography - atmospheric pressure chemical ionization-tandem mass spectrometry (LC/APCI-MS/MS) methods, after solid phase extraction (SPE). More specifically, 3-mL aliquots of urine of exposed individuals (workers or volunteers) along with that of non-occupationally exposed subjects were transferred into glass tubes and spiked with 125 µL of THPI-d internal standard (250 µg/L). Extraction of THPI and PI was then performed using Oasis® SPE cartridges (Waters, Montreux, Switzerland). The

cartridges were first conditioned with 8 mL of dichloromethane, followed by 8 mL of methanol and 12 mL of water. The urines were passed through the column and discarded. The cartridges were washed with 1.5 mL of 5% methanol: 95% water (v/v) and left to dry for 15 min under vacuum (10 inHg). The analytes were then eluted from the column with 4 mL of dichloromethane into 5 mL glass tubes. The solvent was evaporated to dryness under a gentle nitrogen flow at 40°C. The residues were resuspended in 500 µL of methanol and transferred to vials for LC/APCI-MS/MS analysis.

Analysis of THPI and PI in plasma

Analysis of THPI and PI in blood of exposed individuals was performed as described for urine, except that a 2-mL aliquot of plasma was used and a step for the denaturation of proteins was added, prior to SPE extraction. This step consisted of adding 4 mL of saturated ammonium sulphate to the plasma sample, which was then centrifuged at 3400 rpm for 20 min at 15°C.

Instrumental analysis

Apparatus

Analysis of THPI and PI was performed using a Varian Model 212-LC Binary Gradient LC system (Les Ulis, France) connected to a Prostar model 410 autosampler (Varian, Les Ulis, France) and coupled to a Model 1200 L quadrupole MS (Varian, Les Ulis, France) operating in APCI mode. The APCI interface was operated in the negative ion mode and its specific APCI-MS parameters were manually optimized and identical for both analytes.

Analytical conditions

The compounds were separated using a C_{18} Zorbax Eclipse Plus column (4.6 x 150 mm, 3.5 µm) from Agilent (Morges, Switzerland). The temperature of the column was maintained at 30°C. The mobile phase consisted of: eluent A composed of 90% water and 10% acetonitrile (9:1), and eluent B of 10% water and 90% acetonitrile (1:9). For THPI analysis, elution was performed in 14 min using a solvent gradient, at a flow rate of 0.8 mL/min. The following solvent program was used: 90% eluent A for 3 min, followed by a linear gradient to 5% eluent A from 3 to 10 min, maintained at 5% eluent A from 10-13 min before returning to initial conditions of 90% eluent A in 1 min. For PI analysis, elution was performed in 21 min at a flow rate of 0.8 mL/min using the following sequence: 90% eluent A for 15 min and then ramping to 5% eluent A in 30 sec for a 4 min clean up at 5% eluent A prior to returning to initial conditions in 1 min. Several gradients were tested to obtain a chromatographic run as short as possible while allowing separation from interfering peaks and aiming at the best sensitivity for both compounds. In addition, we opted for 12-min re-equilibration period between runs. The samples were kept at 8°C on the injection tray and 10 µL were injected.

Once analytes and mobile phase reached the ionization source, they were subjected to a needle voltage of -10 µA, a spray shield voltage of -600 V and a spray chamber temperature of 60°C. Then, air as nebulizer gas was set to a pressure of 60 psi followed by vaporizing gas of 12 psi with a temperature of 500°C. Lastly, a drying gas (N_2) was set to 35 psi with a temperature of 350°C in the hexapole before product ions were transmitted in quadrupoles. In the collision cell, ions were fragmented with argon at a pressure of 1.45 mTorr on average. Collision energy (CE) was 19.5 V for THPI, 22.5 V for THPI-d and 5V for PI. The precursor and product ion analyzed

were *m/z* 149.4/95.6 for THPI, *m/z* 156.1/95.6 for THPI-d and *m/z* 145.8/145.8 for PI (PI not fragmented). Fragment ions were then detected by the electron multiplier voltage at 1455 V. Identification and quantification were performed in multiple reaction monitoring (MRM) mode.

Quantification of THPI and PI

The quantification of THPI and PI was obtained from standard calibration curves in urine or in plasma. These latter curves were established by plotting the response factors as a function of the concentrations levels, over a maximum range of 1 to 250 µg/L for both analytes depending on the matrix. The response factors corresponded to the peak-area ratios of each compound ion to the internal standard ion.

Methods validation

Since no commercial quality control samples was available for THPI and PI, the criteria used to validate the four methods were specificity, sensitivity, linearity, intra- and inter-day precision, accuracy, recovery and stability.

Specificity

Specificity was determined by analyzing ten urine and plasma samples from unexposed individuals and by verifying the absence of endogenous interferences on the chromatograms for these blank matrices.

Sensitivity

Sensitivity of the analytical methods was estimated by the limit of detection (LOD) and the limit of quantification (LOQ). LOD was calculated from calibration curves (6 for urines and 10 for plasma) using the definition:

$y - y_B = 3s_B$

where y was the response factor, y_B was the blank response or the intercept of regression line, and s_B the standard deviation of the blank [19]. The LOD of each compound was determined for each calibration curve, and then means were calculated and considered as LODs. As for the LOQ, it was defined as equivalent to 3.3 times the LOD.

Linearity

Linearity of the methods was evaluated by calculating regression parameters of calibration curves in urine or in plasma for both compounds by the least square fit method. Results were expressed using the average coefficient of determination (R^2) of eight calibration curves in urine and ten calibration curves in plasma over the studied ranges.

Intra- and inter-day precision and accuracy

Intra-day and inter-day variations were assessed by the precision and the accuracy of replicates of three different levels of spiking of blank urine with THPI and PI and of five levels of spiking of blank plasma. The intra-day variation was measured by the repeatability of each level prepared in two replicates and analyzed during the same day (n = 6), and the inter-day variation was assessed by the repeatability of each level (daily prepared) on eight consecutive days for urine (n = 8) and twenty consecutive days for plasma (n = 20). Precision was expressed as a percentage of relative standard deviation (% RSD) and defined as the ratio of standard deviation to mean of the

response factor for each level of spiking multiplied by 100. Accuracy (expressed in percentage) was estimated by the following equation:

$$\frac{\left(\text{average measured amount - known spiking amount}\right)}{\text{known spiking amount}} \times 100$$

Recovery

To determine the recovery of each compound in both studied matrices after extraction, the ratio of measured amount of THPI and PI in extracts to the theoretical spiked amount in blank urine or plasma was calculated for each level. The recovery was expressed as a percentage.

Stability

To establish stability of compounds in urine and in plasma, pools of blank urine or plasma were spiked at different concentration levels defined above for calibration curves, and then separated in aliquots and stored at -20°C. Every day, over an 8-day period for urine and a 20-day period for plasma, an aliquot of each concentration level was thawed and analyzed.

The stability of urinary samples from volunteers exposed to captan or folpet was also tested. Two samples thawed and analyzed for THPI or PI in a given run were kept at 4°C and processed and reanalyzed during the following run.

Application of the methods

The use of the analytical methods for the quantification of THPI and PI in human plasma and urine samples of exposed individuals was then tested. THPI and PI concentrations were measured in the urine and plasma collected repeatedly over a 96- and 72-h period respectively following an

oral and dermal exposure to captan in ten volunteers. These metabolites were also measured in urine samples repeatedly collected over a typical workweek in applicators exposed to captan and folpet.

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 written informed consent, and were acquainted with the risks of participating and their right to withdraw from the study at all time.

Results and discussion

The analytical methods developed allowed to accurately, specifically and sensitively quantify THPI and PI in both human urine and plasma by LC/APCI-MS/MS.

Methods development

In developing the methods, several tests were performed to determine the best sample treatment and analysis conditions for a precise, reliable, easy and quick procedure. First, liquid-liquid extraction of THPI and PI analytes in plasma and urine using acetonitrile or dichloromethane solvents were tested, but solid phase extraction (SPE) was shown to provide noticeably improved processing recoveries and reproducibility, less interfering peaks on PI chromatograms and simpler and faster processing of samples (data not shown). Once chromatographic conditions were properly set, it was also evaluated whether acid or enzymatic hydrolyses were needed to deconjugate metabolites in human plasma and urine since interactions between thiol- and non-thiol-containing proteins and captan or folpet were reported [20]. This procedure was found unnecessary and even lead to some degradation of THPI and PI, as assessed on urine samples from volunteers orally exposed to captan or folpet after incubation at 37^{o}C with β-glucuronidase/arylsulfatase or heating at 100^{o}C in the presence of HCl 12 N during different time periods (2, 4, 6 and 16 h) (data not shown). Analysis was thus performed without any hydrolysis step.

For LC-MS separation and quantification of THPI and PI, different polar solvents and mixed solutions were also tested as mobile phases. Acetonitrile was finally selected instead of methanol because sensitivity was significantly increased and peak separation was better defined for PI, which fragmentation was not achieved. Several analytical columns were further tested (*i.e.*, Luna® C_{18}, Luna® NH_2 columns from Phenomenex, Spherisorb ODS2 from Waters and C_{18} Zorbax Eclipse Plus from Agilent), but since THPI is a weak base (pKa = 9.65), PI a weak acid (pKa = 6.96) and both are polar molecules, the C_{18} Zorbax Eclipse Plus column was found to be the most appropriate to retain these two metabolites and the internal standard. Thus, short retention times were obtained with a very good repeatability for all analytes: 5.8 minutes for THPI, 8.7 minutes for PI and 5.6 minutes for THPI-d. In addition, electrospray ionization (ESI) was initially selected prior to APCI mode, but no peak was observed for PI and the sensitivity for THPI was poor.

Chromatography

Figures 2-5 show chromatograms of plasma and urine samples of non-occupationally exposed individuals along with blank plasma and urine samples spiked with different concentrations of THPI or PI following treatment of samples by solid phase extraction (see Materials and Methods). Typical chromatograms of plasma and urine samples of volunteers treated with captan and folpet are also represented. For THPI and THPI-d quantification, clean chromatograms were observed with limited background interference, implying that clean up treatment of samples was efficient and analytical conditions were suitable for the methods to be specific. Moreover, the formation of fragment ions (Fig. 2 and Fig. 4) facilitated identification of both analytes, which were also used for quantification. On the other hand, for PI quantification, chromatograms contained several peaks other than those of PI and THPI-d used as an internal standard (Fig. 4

and 5) and fragmentation of PI was not achieved such that only the parent ion was quantified. To separate interference peaks from that of PI, an optimization of LC separation was performed and a high percentage of water in the eluent phase was needed. Different analytical conditions were thus required to analyze PI and THPI. However, both conditions were accurate for analyzing THPI-d, although THPI elution conditions provided narrower peaks and better sensitivity.

Analysis of chromatograms of plasma and urine samples of individuals of the general population non-occupationally exposed to captan or folpet also shows the presence of a baseline level of exposure in some cases. As illustrated in Fig. 2b, Fig. 4a and Fig. 5a, THPI and PI was detectable in some urine samples of non exposed individuals and also in plasma for PI, but in very small concentrations (about 10 nmol/L for THPI in urine, and on average 5.5 nmol/L in plasma and 4 nmol/L in urine for PI). Barr *et al.* [3] detected also THPI in 43% of plasma samples from 70 non-exposed women living in an urban environment, and 51% of samples contained PI.

Chromatograms of plasma and urine samples of volunteers orally exposed to captan and folpet, with and without spiking with reference standards, were further compared (Fig. 2c, Fig. 3c, Fig. 4c and Fig. 5b). For both THPI and PI, no chromatographic differences were noted between spiked and non-spiked matrices; retention times were exactly the same, peaks had the same shape and no interference peaks appeared. These methods thus appeared specific for the biomonitoring of exposure in individuals.

Quantification

To quantify THPI or PI, peak area ratio of the analyte to the internal standard was calculated for the various concentration levels of the calibration curves; this adjustment by internal standard

peak area allowed to account for analyte loss in extraction and analysis, as well as eliminate potential variations due to the apparatus and reduce errors associated with measurements. The use of a deuterated form of THPI as an internal standard also provided a molecule structurally related to captan and folpet biomarkers, but distinguishable by its mass. This increased the precision and specificity of the methods, even for PI, which had several similarities with THPI despite its aromatic core.

Methods validation

The methods developed were evaluated using the following criteria described in Materials and Methods section: sensitivity, linearity, intra- and inter-day precision and accuracy, recovery and stability.

Table 1 presents the estimated LODs and LOQs of THPI and PI in urine and plasma. They were established within the concentration range of 0.5 to 50 µg/L for THPI and PI in urine and 1 to 100 µg/L for THPI and PI in plasma. LOD was 0.58 and 1.47 µg/Lfor THPI in urine and plasma respectively, and corresponding LOQ was 1.90 and 4.87 µg/L. In comparison, LOD was 1.14 and 2.17 µg/L for PI in urine and in plasma respectively, and LOQ was 3.75 and 7.19 µg/L. The sensitivity was however better for THPI than PI given that the latter molecule could not be fragmented in MS and elution conditions had to be adjusted to provide a better separation of PI from interfering peaks on the chromatograms. The developed methods for both THPI and PI were at least as sensitive as those published in the literature (Table 2). For instance, our values were comparable to the method of Barr *et al.* [3] and Hines *et al.* [6].

As for the linearity of standard calibration curves in methanol, plasma and urine, they are displayed in Fig. 6 for THPI and in Fig. 7 for PI. All curves were linear over the studied concentration range for THPI and PI, and resulted in a coefficient of determination greater than 0.99. Furthermore, matrix effects were not tested *per se* since APCI mode is less susceptible than ESI mode [18; 20; 21]. However, for THPI, calibration curves prepared in methanol had similar slopes to those prepared in urine, indicating the absence of urinary matrix effect for this analyte. On the other hand, matrix effects were obvious for calibration curves of THPI and PI prepared in plasma as well as those of PI in urine. Calibration was thus performed using standard curves in plasma and urine for both analytes (with more spiking levels for plasma given the observed wider concentration range of THPI and PI in volunteers and more calibration curves for validation given plasma matrix effects).

Precision and accuracy of the methods of analysis of THPI and PI in plasma and urine are further presented in Table 3. Intra-day and inter-day precision and accuracy were satisfactory with % relative standard deviation (RSD) less than 15% (except for the first urinary level of THPI and PI) and % relative error (RE) of less than 20%.

Table 3 gives also an overview of recoveries of THPI and PI in spiked plasma and urine samples, after solid-phase extraction and processing. They were quite good for both compounds in these matrices, except for PI at low concentrations given matrix effects. The mean (± SD) recoveries of THPI (i.e. all spiked sample results combined) were 90.4 ± 9.9% (n = 20) in urine and 90.9 ± 6.7% (n = 100) in plasma; corresponding values for PI were 72.9 ± 18.6% (n = 20) in urine and 79.6 ± 11.3% (n = 100) in plasma (as plasma was not collected for workers, more runs with urine samples were performed than with plasma samples). These results were similar to those reported

in the literature. Indeed, several authors found a recovery rate of THPI in human urine varying between 82 to 90% over the same concentration range [6-10]; Barr *et al.* [3] obtained a recovery rate of 91% for THPI in human plasma, and of 89% for PI in human plasma like Canal-Raffin *et al.* [4] in rat plasma (Table 2). To our knowledge, no published methods are available for the analysis of PI in human urine.

Furthermore, stability of THPI and PI standards in plasma and urine samples kept at -20°C was tested over an 8-day period for urine and over a 20-day period for plasma. Since intra-day precision and accuracy values were similar to those of inter-day precision and accuracy, both analytes were thus considered stable in plasma and urine over the studied period (Table 3). The same observations were made by Canal-Raffin *et al.* [4] for the stability of PI in rat plasma, and Barr *et al.* [3] reported that THPI and PI in human plasma were stable over a period of four months.

THPI and PI in urine samples of volunteers treated with captan or folpet were also relatively stable, when kept at 4°C and re-analyzed in the following analytical run. A coefficient of variation ranging between 2.98 and 6.57% were obtained for THPI (n = 12) and between 1.51 and 7.56% for PI (n = 12). The same tests could not be performed with plasma samples because of the limited amount of matrix available.

Application

With the developed analytical methods, it was verified that THPI and PI could easily be quantified in urine and plasma of volunteers exposed to captan or folpet at 1 mg/kg of body weight by oral route and at 10 mg/kg of body weight by dermal route; Fig. 8 presents the mean

concentration-time profiles of THPI and PI in urine and plasma following both an oral and dermal exposure. The methods were found to be sensitive enough to document the toxicokinetics of THPI in human plasma and urine at equivalently realistic daily absorbed doses in workers.

On the other hand, PI concentrations in plasma and urine were much lower than those of THPI for the same exposure dose. When PI was administered to rats, Chasseaud *et al.* [23] observed that about 80% of the dose was metabolized and excreted in urine as phthalamic acid and 7% as phthalic acid, showing that PI is a minor metabolite of folpet in urine. Moreover, Chasseaud *et al.* [24] established that when labelled ^{14}C-folpet was administered to rats, 80% was recovered in the urine as phthalamic acid, and Canal-Raffin *et al.* [4] observed that PI was rapidly hydrolyzed to phthalamic acid in plasma following an intratracheal administration of folpet in rats. PI is however more specific to folpet exposure than the phthalic acid, which is also a phthalate metabolite [25; 26].

Table 4 presents pre-and post-shift levels of THPI and PI in urine during the course of a workweek in applicators exposed to captan and folpet, respectively. This table shows that the metabolites were easily measured in the urine of workers following a one-day exposure episode, as compared to pre-seasonal measurements or, in the case of folpet, baseline values observed a few days after application. According to our results, THPI appears as a sensitive and specific biomarker of captan exposure in exposed individuals; PI can also be considered as a specific biomarker of folpet exposure but is less sensitive than THPI.

Conclusion

The LC/APCI-MS/MS methods proved to be simple and reliable to quantify THPI and PI in human plasma and urine. Their good sensitivity, specificity, linearity, accuracy and precision were also validated and found at least equivalent to methods reported in the literature. These methods thus appear suitable for the biomonitoring of exposure to captan and folpet in exposed individuals.

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Table 1

Performance parameters of the methods

Analyte	Matrix	LOD[a] (mean ± SD) (µg/L)	LOQ[b] (mean ± SD) (µg/L)	Working range (µg/L)
THPI	Urine (*n* = 6)	0.58 ± 0.28	1.9 ± 0.92	0.5 – 50
	Plasma (*n* = 10)	1.47 ± 0.69	4.87± 2.28	1 – 100
PI	Urine (*n* = 6)	1.14 ± 0.22	3.75 ± 0.72	0.5 – 50
	Plasma (*n* = 10)	2.17 ± 0.39	7.19 ± 1.29	1 – 100

[a] Limit of detection (LOD) was calculated from calibration curves (6 for urine and 10 for plasma) using the definition: $y - y_B = 3s_B$, where y was the response factor, y_B was the blank response or the intercept of regression line, and s_B the standard deviation of the blank [19]. The LOD of each compound was determined for each calibration curve, and then means were calculated and considered as LODs.

[b] Limit of quantification (LOQ) was defined as 3.3 times the detection limit.

Table 2

Comparison of performance parameters of available analytical methods for THPI and PI analysis in human plasma and urine.

Methods	Detection technique	LOD[a]	Spiking	Recovery (%) (mean ± SD)	Extraction type
THPI in urine					
Shoen *et al.* [9]	GC/NPD/MS	165 nmol/L	198 nmol/L	82 ± 10.5 (*n* = 9)	Solvent extraction
			331 nmol/L	87 ± 5.2 (*n* = 5)	
			3307 nmol/L	86 ± 5.8 (*n* = 5)	
van Welie *et al.* [10]	GC/CI/MS	17.9 nmol/L	17.9 – 360 nmol/L	54 ± 5.0 (*n* = 4)	Solvent extraction
Krieger and Thongsinthusak [7]	GC/NPD/MS	33.1 – 66.1 nmol/L	66.1 nmol added	90	Solvent extraction
			661 nmol added	95	
Krieger and Dinoff [8]	GC/ECD/MS	33.1 nmol/L	33.1 – 13230 nmol/L	78 ± 5.0	Solvent extraction
Hines *et al.* [6]	GC/MS	11.2 nmol/L	33.1 – 265 nmol/L	86 ± 9.1 (*n* = 179)	Solvent extraction
Our study	LC/APCI-MS/MS	3.82 nmol/L	3.31 – 33.1 pmol added	94.3 ± 7.2 (*n* = 24)	Solid phase extraction
THPI in plasma					
Barr *et al.* [3]	GC/HR/MS	1 pg/g	1.58 – 2646 nmol/L	91 ± 8.0 (*n* = 6)	Solid phase extraction
Our study	LC/APCI-MS/MS	9.76 nmol/L	16.5 – 827 pmol added	90.9 ± 6.7 (*n* = 100)	Solid phase extraction

Methods	Detection technique	LOD[a]	Spiking	Recovery (%) (mean ± SD)	Extraction type
PI in Plasma					
Barr *et al.* [3]	GC/HR/MS	20 pg/g	1.58 – 2646 nmol/L	89 ± 6.0 (*n* = 6)	Solid phase extraction
Canal-Raffin *et al.* [4]	HPLC-UV/DAD	33.1 nmol/L	67.9 – 679.7 nmol/L	90 ± 6.9 (*n* = 12)	Solid phase extraction
Our study	LC/APCI-MS/MS	7.72 nmol/L	17.0 – 850 pmol added	79.6 ± 11.3 (*n* = 100)	Solid phase extraction

n: Number of samples.

GC/CI/MS: gas chromatograph equipped with a chemical ionization and a mass spectrometer; GC/ECD/MS: gas chromatograph with an electrolytic conductivity detector and a mass spectrometer; GC/HR/MS: gas chromatography-high resolution mass spectrometry; GC/NPD\MS: gas chromatograph equipped with nitrogen-phosphorus detector and a mass spectrometer; HPLC-UV/DAD: high-performance liquid chromatography with ultraviolet diode array; LC/APCI-MS/MS: liquid chromatography with a mass spectrometer and atmospheric pressure chemical ionization.

[a] Limit of detection (LOD) was defined as the concentration with a signal-to-noise ratio of at least 3, except for Barr *et al.* [3] and our study, where the LOD was calculated as $3s_0$ where s_0 was estimated as the *y*-intercept of a linear regression analysis of a plot of the absolute standard deviation versus the concentration.

Table 3

Recovery, intra- and inter-day precision and accuracy of THPI and PI at three different spiking levels (pmol added) of blank human urine and at five different spiking levels (pmol added) of blank human plasma.

Analytes	Matrix	Amount added (pmol)[c]	Recovery[d] (%)	RSD[e] (%)	Intra-day variation[a]			Inter-day variation[b]		
					Amount found[f] (pmol) (mean ± SD[g])	Precision (% RSD[e])	Accuracy[h] (%)	Amount found[f] (pmol) (mean ± SD[g])	Precision (% RSD[e])	Accuracy[h] (%)
THPI	Urine		(*n* = 8)		(*n* = 6)			(*n* = 8)		
		3.31	103.6	15.06	3.58 ± 0.42	11.74	8.13	3.66 ± 0.61	16.69	10.55
		13.2	87.35	13.35	11.50 ± 0.38	3.28	–13.07	11.56 ± 1.54	13.35	–12.65
		33.1	93.47	9.68	31.58 ± 1.46	4.62	–4.52	30.92 ± 2.99	9.67	–6.53
	Plasma		(*n* = 20)		(*n* = 10)			(*n* = 20)		
		16.5	83.02	13.67	13.78 ± 0.97	7.03	–16.71	13.73 ± 1.88	13.67	–16.98
		33.1	82.92	13.28	27.46 ± 2.75	10.00	–16.98	28.28 ± 4.44	15.70	–14.51
		165	93.44	10.81	155.05 ± 9.07	5.85	–6.25	154.53 ± 16.70	10.81	–6.56
		331	95.32	7.99	316.14 ± 19.23	6.08	–4.42	315.30 ± 25.18	7.99	–4.67
		827	99.49	7.77	818.61 ± 38.07	4.65	–1.01	822.72 ± 63.95	7.77	–0.51

PI	Urine		(*n* = 8)		(*n* = 6)			(*n* = 8)		
		3.40	42.65	6.54	1.42 ± 0.38	21.92	----- [i]	1.24 ± 0.36	28.92	----- [i]
		13.6	82.32	15.4	12.06 ± 0.36	3.03	–11.26	12.9 ± 1.06	8.19	–4.51
		34.0	88.73	14.29	31.01 ± 7.09	14.4	-8.73	33.89 ± 8.18	8.18	–0.26
	Plasma		(*n* = 20)		(*n* = 10)			(*n* = 20)		
		17.0	67.3	15.2	11.42 ± 0.84	7.35	----- [i]	9.80 ± 0.85	8.67	----- [i]
		34.0	73.4	14.3	25.24 ± 2.01	9.41	----- [i]	25.82 ± 2.23	8.65	----- [i]
		170	87.3	13.5	151.84 ± 14.30	11.41	–10.64	148.38 ± 17.16	11.56	–12.68
		340	85.1	10.1	277.48 ± 12.96	4.67	–18.35	277.60 ± 15.67	5.64	–18.31
		850	90.4	12.8	800.23 ± 74.62	9.32	–5.81	748.81 ± 96.99	12.95	–11.86

n: Number of samples for each spiking level.

[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 an 8-day period for urine and a 20-day period for plasma.

[c] Amount of THPI or PI (expressed in pmol) added to blank urine or plasma at the different concentration levels.

[d] Percent recovery of THPI or PI amounts (different levels) added to blank urine or plasma samples and processed as described in Materials and Methods.

[e] Precision or RSD: relative standard deviation for *N* replicates calculated as (SD/mean) x 100.

[f] Recovered amount of THPI or PI after sample processing and calculated from calibration curves.

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

[h] Accuracy, or percent relative error, calculated using the following equation: (amount found – amount added)/amount added x 100.

[i] These accuracy values are not reported since matrix effects were apparent for PI at these low spiking levels contrary to THPI-d used as an internal standard.

Table 4

Concentrations of THPI and PI in applicators exposed to captan and folpet, respectively, during the course of a typical workweek along with pre-seasonal concentrations

Metabolite analyzed	Applicator	Pre-seasonal	Concentration (nmol/L)									
			Workday 1[a]		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
THPI												
	Applicator 1	<LOD[b]	4.24	14.17	23.61	43.18	10.01	11.27	12.07	12.93	10.62	5.83
	Applicator 2	<LOD	<LOD	21.53	54.17	43.09	43.71	17.55	15.38	14.16	8.55	10.17
PI												
	Applicator 1	<LOD[c]	16.95	11.41	8.42	17.01	8.87	<LOD	<LOD	<LOD	<LOD	17.74
	Applicator 2	≈LOD	21.71	42.43	42.61	31.51	33.97	26.19	≈LOD	<LOD	13.86	21.70
	Applicator 3	<LOD	<LOD	13.71	15.06	<LOD	<LOD	<LOD	<LOD	<LOD	8.96	≈LOD

[a] Captan and folpet were sprayed on workday 1 only.

[b] LOD of THPI in urine = 3.82 nmol/L.

[c] LOD of PI in urine = 7.72 nmol/L.

Figure captions

Fig. 1 Chemical structures of (**a**) captan and its metabolite THPI and (**b**) folpet and its metabolite PI.

Fig. 2 Representative chromatograms of THPI and THPI-d in human urine: (**a**) blank urine spiked with 10 µg/L of THPI (1) and 75 µg /L of THPI-d (2); (**b**) urine from an unexposed subject (3) and blank urine spiked with 1 µg /L of THPI (4); (**c**) blank urine spiked with 10 µg/L of THPI (1) and 75 µg /L of THPI-d (2) along with a urine sample of a volunteer exposed orally to captan (5) spiked with 75 µg /L of THPI-d (6). Chemical structures of THPI, THPI-d and their measured ion fragment are also represented.

Fig. 3 Representative chromatograms of PI and THPI-d in human urine: (**a**) blank urine spiked with 1 µg /L of PI (1) and 26.5 µg /L of THPI-d (2); (**b**) urine from an unexposed subject (3) and blank urine spiked with 10 µg /L of PI (4), 50 µg /L of PI (5) and 100 µg /L (6); (**c**) blank urine spiked with 1 µg /L of PI (1) and 26.5 µg /L of THPI-d (2) along with a urine sample of a volunteer exposed orally to folpet (7) spiked with 26.5 µg /L of THPI-d (8). Chemical structures of PI, THPI-d and its measured ion fragment are also represented.

Fig. 4 Representative chromatograms of THPI and THPI-d in human plasma: (**a**) blank plasma spiked with 50 µg /L of THPI (1) and 79.5 µg /L of THPI-d (2); (**b**) plasma sample from an unexposed subject (3) and blank plasma spiked with 1 µg /L of THPI (4); (**c**) blank plasma spiked with 50 µg /L of THPI (1) and 79.5 µg /L of THPI-d (2) along with a plasma sample of a volunteer exposed orally to captan (5) spiked with 79.5 µg /L of THPI-d (6).

Fig. 5 Representative chromatograms of PI and THPI-d in human plasma: (**a**) plasma sample from an unexposed subject (1) spiked with 79.5 µg /L of THPI-d (2) and blank plasma spiked with 1 µg /L of PI (3) and 79.5 µg /L of THPI-d (4); (**b**) plasma from an unexposed subject (5) and blank plasma spiked with 10 µg /L of PI (6); (**c**) blank plasma spiked with 1 µg /L of PI (3) and 79.5 µg /L of THPI-d (4) along with a plasma sample of a volunteer exposed orally to folpet (7) spiked with 79.5 µg /L of THPI-d (8).

Fig. 6 Calibration curves of THPI over the concentration range of 0.003 to 0.66 µmol/L (0.5 to 100 µg/L) prepared in methanol (▼), blank urine (○) and blank plasma (●). The linearity of curves is described by the coefficient of determination (R^2) and equations are presented.

Fig. 7 Calibration curves of PI over the concentration range of 0.5 to 50 µg/L prepared in methanol (▼), blank urine (○) and blank plasma (●). The linearity of curves is described by the coefficient of determination (R^2) and equations are presented.

Fig. 8 (**a**) Concentration-time profiles of THPI (mean ± SD) in volunteers exposed to captan orally (1 mg/kg) or dermally (10 mg/kg) and (**b**) concentration-time profiles of PI (mean ± SD) in volunteers exposed orally and dermally to folpet (1 and 10 mg/kg, respectively). (-●-) Urinary excretion profile following oral exposure; (-○-) urinary excretion profile following dermal exposure; (-▼-) plasma profile following oral exposure; (-Δ-) plasma profile following dermal exposure.

Figure 1

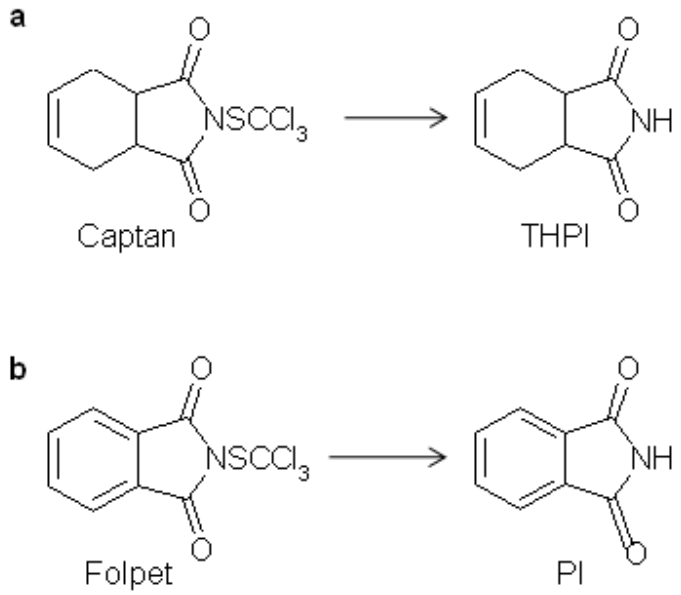


Figure 2

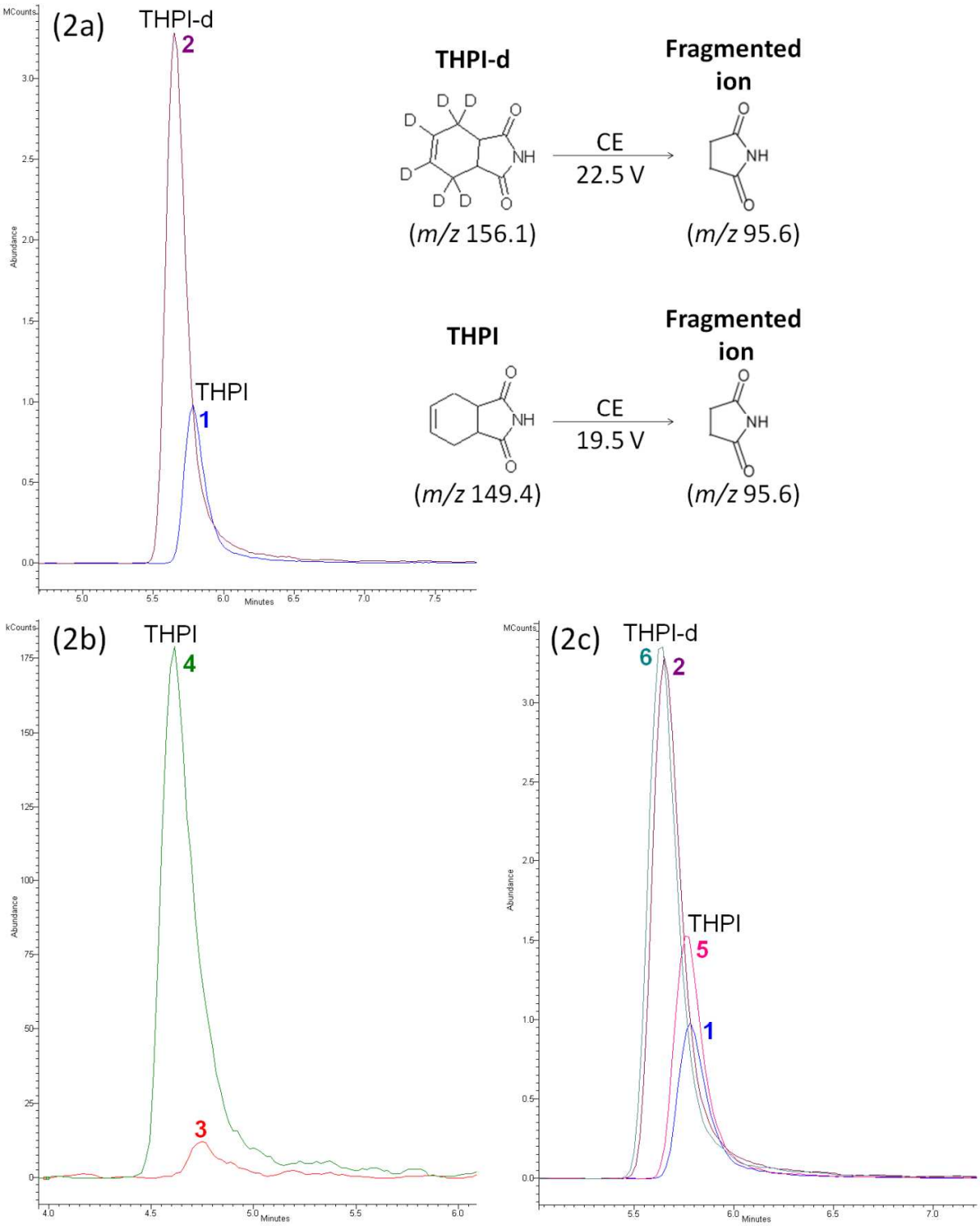

(2a)
THPI-d
2
THPI
1
MCounts
Abundance
Minutes
THPI-d
Fragmented ion
CE
22.5 V
(m/z 156.1)
(m/z 95.6)
THPI
Fragmented ion
CE
19.5 V
(m/z 149.4)
(m/z 95.6)
(2b)
THPI
4
3
kCounts
(2c)
THPI-d
6
2
THPI
5
1

Figure 3

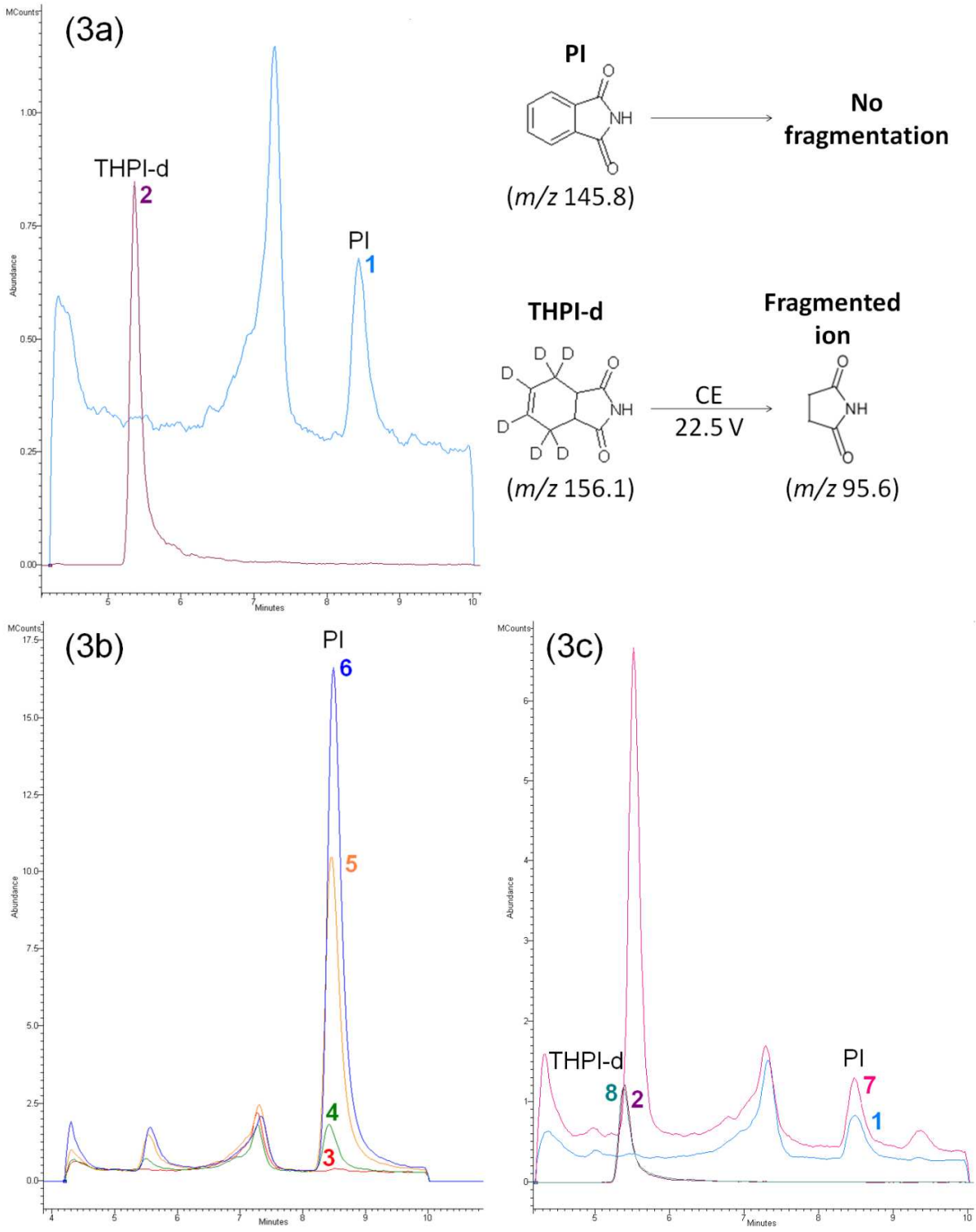

(3a)
MCounts
Abundance
Minutes
THPI-d
2
PI
1
PI
NH
No fragmentation
(m/z 145.8)
THPI-d
D
NH
CE
22.5 V
Fragmented ion
(m/z 156.1)
(m/z 95.6)
(3b)
PI
6
5
4
3
(3c)
THPI-d
8
2
PI
7
1

Figure 4

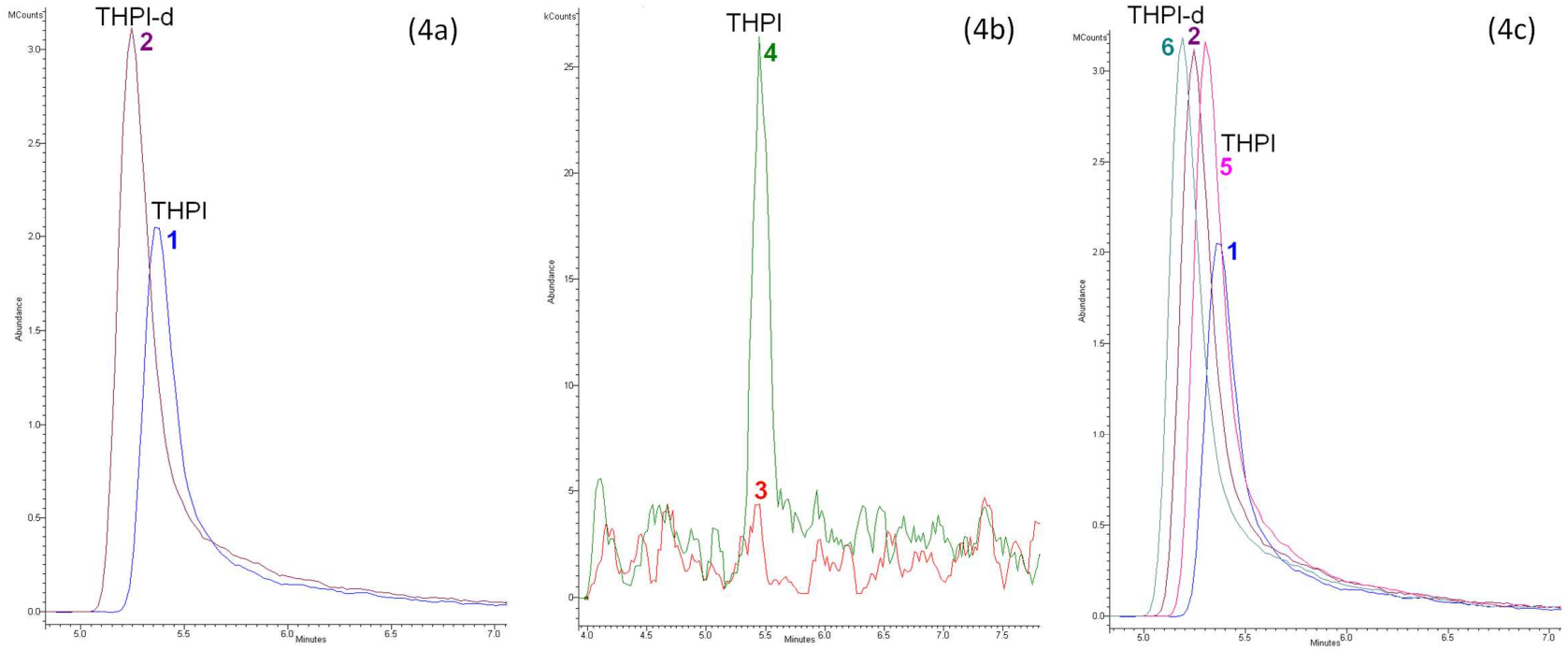

THPI-d
2
THPI
1
(4a)
MCounts
Abundance
Minutes
THPI
4
3
(4b)
kCounts
THPI-d
6
2
THPI
5
1
(4c)

Figure 5

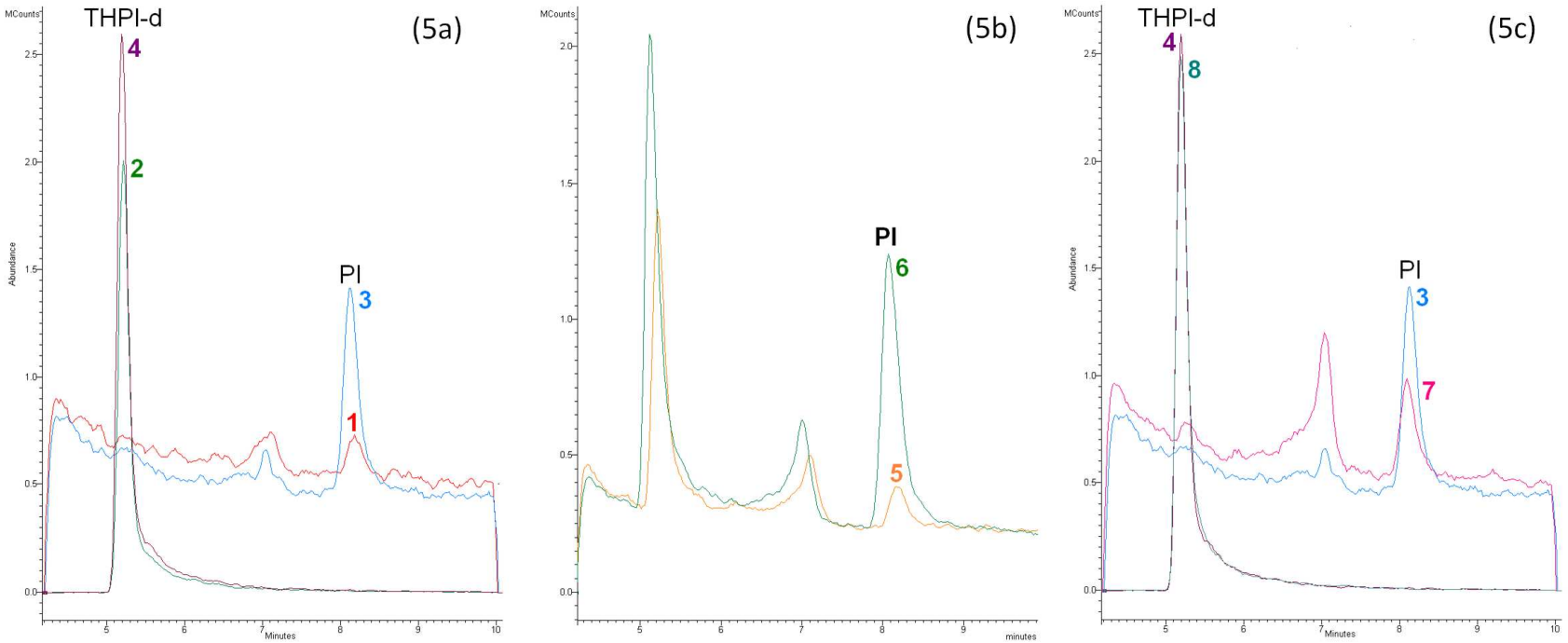

THPI-d
4
2
PI
3
1
(5a)
MCounts
Abundance
Minutes
PI
6
5
(5b)
minutes
THPI-d
4
8
PI
3
7
(5c)

Figure 6

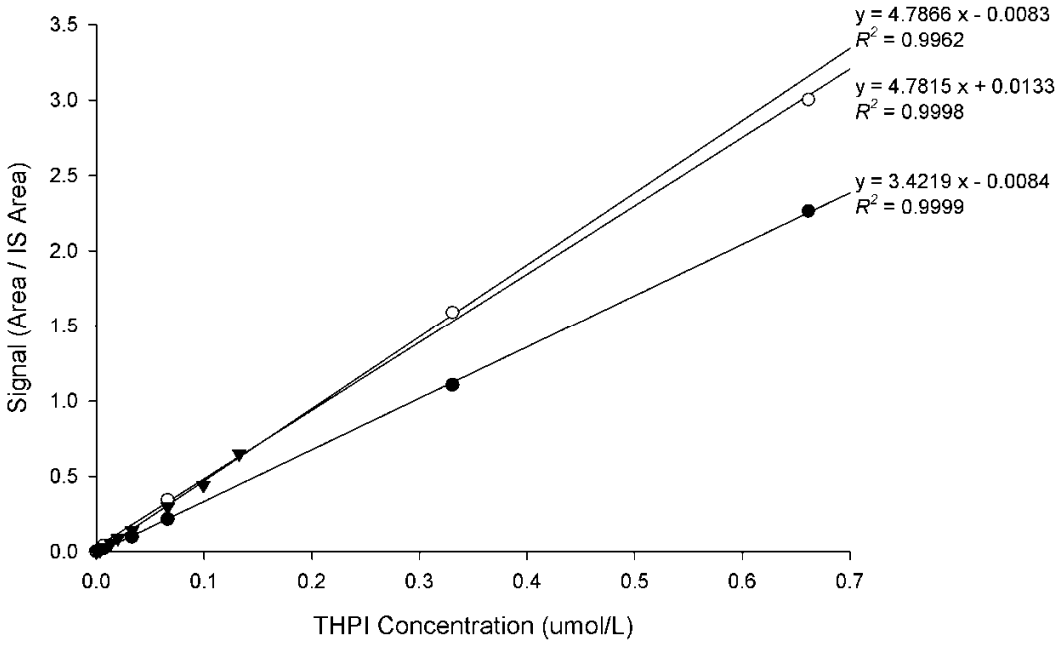

y = 4.7866 x - 0.0083
R2 = 0.9962
y = 4.7815 x + 0.0133
R2 = 0.9998
y = 3.4219 x - 0.0084
R2 = 0.9999
Signal (Area / IS Area)
THPI Concentration (umol/L)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
0.1
0.2
0.3
0.4
0.5
0.6
0.7

Figure 7

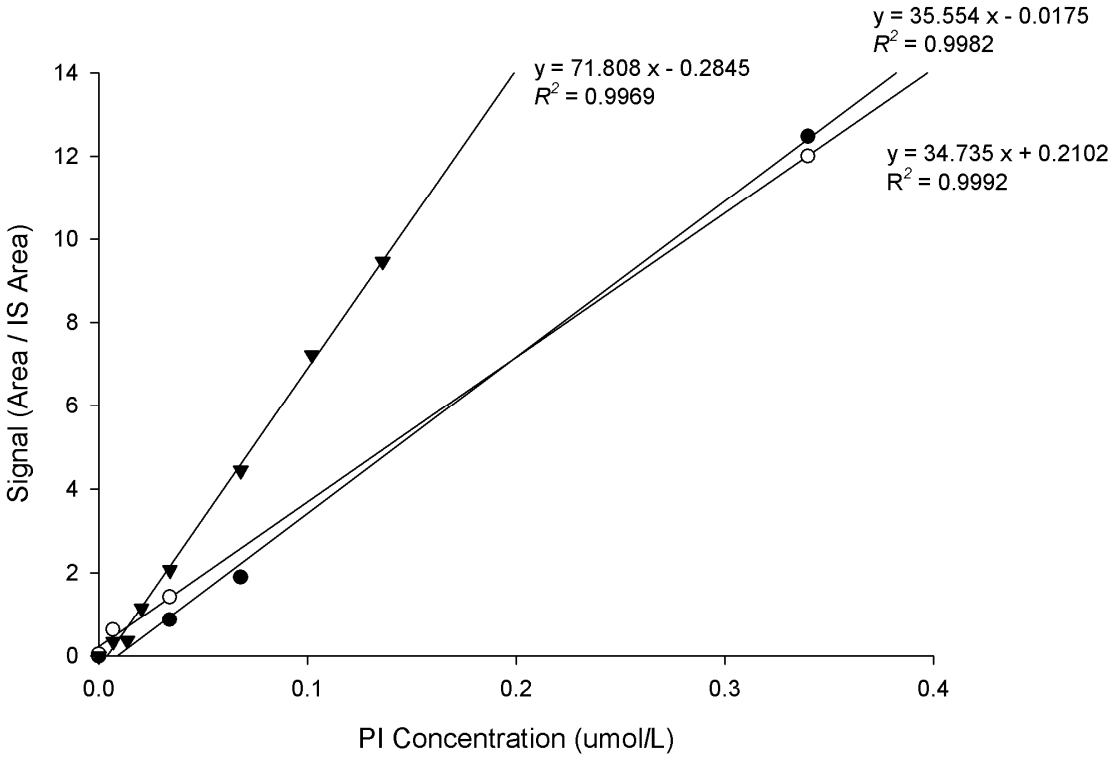
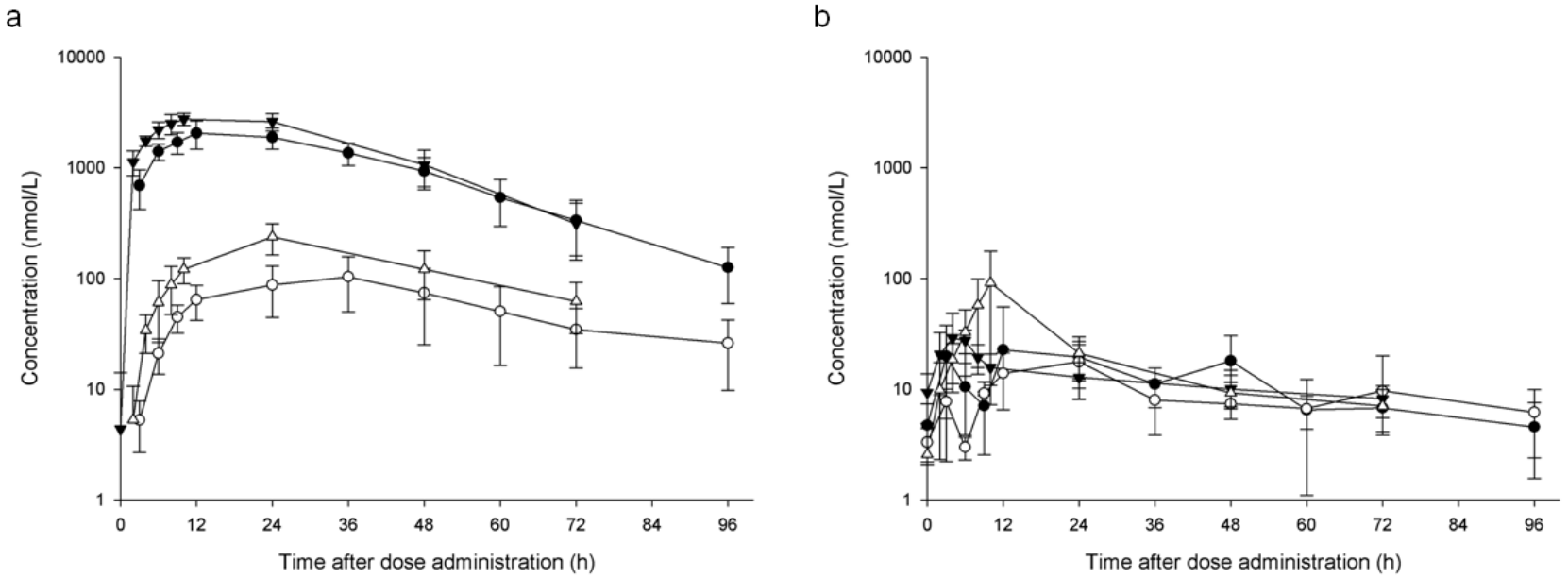

y = 35.554 x - 0.0175
R^2 = 0.9982
y = 71.808 x - 0.2845
R^2 = 0.9969
y = 34.735 x + 0.2102
R^2 = 0.9992
Signal (Area / IS Area)
PI Concentration (umol/L)
0
2
4
6
8
10
12
14
0.0
0.1
0.2
0.3
0.4

Figure 8


a
b
Concentration (nmol/L)
Time after dose administration (h)
10000
1000
100
10
1
0
12
24
36
48
60
72
84
96