Measurement of *p*-Nitrophenol in the Urine of Residents Whose Homes Were Contaminated with Methyl Parathion

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During the last several years, illegal commercial application of methyl parathion (MP) in domestic settings in several U.S. Southeastern and Midwestern States has affected largely inner-city residents. As part of a multiagency response involving the U.S. Environmental Protection Agency (U.S. EPA), the Agency for Toxic Substances and Disease Registry (ATSDR), and state and local health departments, our laboratory developed a rapid, high-throughput, selective method for quantifying *p*-nitrophenol (PNP), a biomarker of MP exposure, using isotope dilution high-performance liquid chromatography–tandem mass spectrometry. We measured PNP in approximately 16,000 samples collected from residents of seven different states. Using this method, we were able to receive sample batches from each state; prepare, analyze, and quantify the samples for PNP; verify the results; and report the data to the health departments and ATSDR in about 48 hr. These data indicate that many residents had urinary PNP concentrations well in excess of those of the general U.S. population. In fact, their urinary PNP concentrations were more consistent with those seen in occupational settings or in poisoning cases. Although these data, when coupled with other MP metabolite data, suggest that many residents with the highest concentrations of urinary PNP had significant exposure to MP, they do not unequivocally rule out exposure to PNP resulting from environmental degradation of MP. Even with their limitations, these data were used with the assumption that all PNP was derived from MP exposure, which enabled the U.S. EPA and ATSDR to develop a comprehensive, biologically driven response that was protective of human health, especially susceptible populations, and included clinical evaluations, outreach activities, community education, integrated pest management, and decontamination of homes. *Key words:* mass spectrometry, methyl parathion, *p*-nitrophenol, urine. *Environ Health Perspect* 110(suppl 6):1085–1091 (2002).
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In the fall of 1994, officials in Ohio discovered that methyl parathion (MP) had been used for domestic pest control, primarily in inner-city homes (*1–3*). Further investigations uncovered that an unlicensed operator had illegally applied MP in hundreds of homes for cockroach control. This event resulted in costly government-subsidized evacuations and home decontaminations (*4,5*).

Since the episode in Ohio, similar incidents have occurred in seven other Southeastern and Midwestern States. To aid in prioritizing evacuation and decontamination efforts, the Agency for Toxic Substances and Disease Registry (ATSDR) asked our laboratory to provide biomonitoring data for residents affected by these illegal applications. We developed a rapid, high-throughput method for quantifying a biomarker of MP exposure and applied this method to assess MP exposure in residents of contaminated homes.

MP is a highly toxic organophosphate insecticide. Since its introduction in 1952, MP has been used primarily for the control of boll weevils on cotton (*6*). Its mode of action, common to all organophosphate insecticides, is the competitive inhibition of acetylcholinesterase, an enzyme responsible for the breakdown of the neurotransmitter acetylcholine (*7,8*). MP is

classified as a restricted-use pesticide by the U.S. Environmental Protection Agency (U.S. EPA) (*6*). It is licensed only for use in outdoor settings, and workers may not reenter fields sprayed with MP for at least 4–5 days (*9*). When applied properly in agricultural settings where it is exposed to water and ultraviolet (UV) light, MP has an environmental half-life of days or weeks (*6,10*). When applied indoors, the environmental half-life may increase substantially.

MP has a biological half-life in animals of 7–17 hr (*11,12*). Upon exposure and subsequent bodily absorption, MP is rapidly metabolized (Figure 1) to biologically active methyl paraoxon. Methyl paraoxon can chemically bind acetylcholinesterase, which can ultimately result in a variety of clinical outcomes including headaches, seizures, respiratory depression, paralysis, and death (*5*). Further metabolism can result in the urinary excretion of dimethylthiophosphate (DMTP), dimethylphosphate (DMP), and *p*-nitrophenol (PNP), either as the free phenol or as its glucuronide or sulfate ester (*13*). The dialkylphosphate metabolites of MP are largely nonspecific because they are potential metabolites of about 75% of the organophosphate pesticides registered with the U.S. EPA

(*14*). Although it is also a metabolite of parathion, nitrobenzene, and a few similar compounds, PNP is considered a more specific biomarker of exposure to MP than the dialkylphosphate metabolites (*3,15*). However urinary PNP can also result from exposure to environmental PNP resulting from the natural environmental degradation of MP or parathion. After an acute exposure, the majority of DMTP, DMP, and PNP is excreted in urine within a few days (*16–19*). However, chronic exposure to MP, as with any nonpersistent organic toxicant, can result in steady-state excretion of the metabolites.

Measurements of the change in acetylcholinesterase activity from an individual's baseline (preexposure) acetylcholinesterase activity is considered the "gold standard" measurement for clinical assessment and management of organophosphate intoxication (*20*). Unfortunately, in nonoccupational exposures, baseline activity levels are difficult to obtain because usually an individual is seen by a clinician after the exposure has occurred. Furthermore, acetylcholinesterase depression measurements typically lack the sensitivity to detect low-level exposures.

Typically, PNP has been quantified in urine as a more sensitive biomarker of exposure to MP or parathion. These methods use
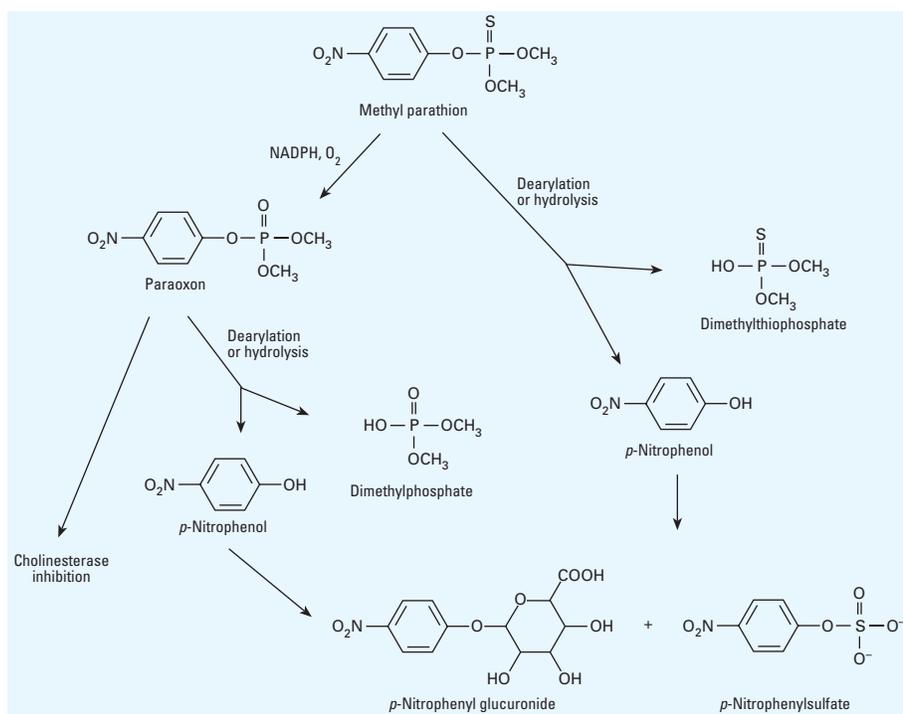
Figure 1. Schematic representation showing the potential metabolic byproducts of MP intoxication. NADPH, nicotinamide adenine dinucleotide phosphate.

high-performance liquid chromatography (HPLC) with UV or diode array detection (21,22) or gas chromatography (GC) with electron capture detection (23) and have limits of detection ranging from 20 to 500 µg/L. These methods lack the selectivity or the sensitivity to measure trace amounts of PNP in urine. More recently, our laboratory reported a multianalyte method for the selective analysis of 12 urinary pesticide metabolites, including PNP, at the low microgram per liter level (24). This method combined a solvent extraction of PNP from urine, a chemical derivatization of PNP to its chloropropyl ether, and a selective analysis using GC–tandem mass spectrometry (GC–MS/MS). The major limitation of this method was the lengthy chemical derivatization step (6 hr) required to adequately analyze for the multiple analytes, which severely limited the analytical throughput of the method. With our multianalyte method, 25 samples could be analyzed in 4 days. This was equivalent to 1 sample per 4 hr. To improve the analytical throughput without sacrificing method sensitivity or selectivity, we opted to use HPLC–MS/MS, allowing us to eliminate the need for chemical derivatization.

We report our high-throughput and selective method for the quantification of urinary PNP. Our new method is capable of analyzing 160 samples in 2 days, or 1 sample every 18 min, representing an almost 13-fold increase in throughput. Using this method, we were able to receive sample batches from state and local health departments; prepare, analyze, and quantify the samples for PNP; verify the results; and report the data to health departments and ATSDR in about 48 hr. We also report data obtained from people exposed to MP or environmental PNP as a result of its illegal residential application in seven Southeastern and Midwestern States. As a result of the epidemic nature of MP exposure in the past several years, we have accumulated what appears to be the largest volume of biological monitoring data for a single pesticide exposure cohort.

Materials and Methods

Materials

All chemicals unless otherwise indicated were obtained in the purest form commercially available from JT Baker Chemical Co. (Phillipsburg, NJ, USA). PNP (gold label 99+% purity) was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA), and ring-$^{13}C_6$-PNP (98% chemical purity, 99.99% isotopic purity) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). The enzyme β-glucuronidase/sulfatase from *Helix pomatia* was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The gases for MS were obtained from Holox (Norcross, GA, USA) or Air Products and Chemicals Inc. (Atlanta, GA, USA) and had a minimum purity of 99.999%. Our solvents, including water, were analytic grade and were obtained from Burdick and Jackson Co.

(Muskegon, MI, USA). All chemicals and solvents were used without further purification. Reagents were prepared with bottled water according to standard laboratory procedures.

Standard Preparation and Characterization

Calibration standards. A PNP stock solution was prepared by dissolving 18 mg PNP in dimethylformamide (DMF). A $^{13}C_6$-PNP stock solution was prepared by dissolving 10 mg ring-$^{13}C_6$-PNP in DMF. The PNP and $^{13}C_6$-PNP stock solutions were combined and diluted with DMF to create eight standard sets whose concentrations encompassed the linear range of the method (10–800 µg/L in urine or 50–2500 ng on column). The stocks and diluted standard sets were stored at –20°C.

Internal standard. A diluted $^{13}C_6$-PNP solution was prepared from the stock such that a 25-µL aliquot gave an approximate 150-µg/L concentration in 3 mL urine. The $^{13}C_6$-PNP concentration was adjusted to be close to the expected or measured value to minimize error. Diluted $^{13}C_6$-PNP standards were divided into aliquots sufficient for two analytical runs and were stored at –20°C until used.

Quality control materials. Quality control (QC) materials were prepared in-house from a base urine pool obtained from multiple donors. The base urine was homogenized, then split into three pools. One pool was not enriched and thus reflected the endogenous, or native, concentration of PNP in the urine. The other two pools were enriched with different levels of PNP. Thus, QC pools with native, low (approximately 75 µg/L), and high (approximately 150 µg/L) concentrations were obtained. All QC pools were mixed thoroughly for 24 hr following enrichment. The pools were filtered through a sterile membrane apparatus (0.2 µm pore size). The filtered urine was dispensed in 20-mL aliquots into sterile bottles. The bottles were capped, labeled, and stored at –20°C until used. All QC pools were characterized to define the mean concentration and the 95th and 99th confidence intervals of PNP by the analysis of at least 20 repeat measurements. QC concentrations were evaluated using standard Westgard multirules (25).

Sample Preparation

Three-milliliter aliquots of urine were spiked with $^{13}C_6$-PNP. The urine samples were diluted with an equal volume of 0.1 M acetate buffer (pH 4.5) containing approximately 430 U/mL β-glucuronidase/sulfatase. The sample tubes were capped and inverted to mix. The urine/enzyme solutions were allowed to incubate at 37°C overnight (about 17 hr) to liberate the PNP from the glucuronides or sulfate esters. The hydrolysates were acidified with 200 µL 3 M HCl to a

pH of approximately 2. Ten milliliters dichloromethane was added to each sample and the tubes were inverted 10 times for extraction. The samples were centrifuged at 2,000 rpm for 15 min to adequately separate the organic and aqueous layers. The organic layers were dried over anhydrous sodium sulfate and transferred to clean sample tubes. As a keeper solvent, 100 μL DMF was added to each extract and they were concentrated to 100 μL using a TurboVap concentrator (Zymark Corporation, Hopkinton, MA) with 15 psi nitrogen head pressure at 37°C. The concentrated extracts were transferred to HPLC autosampler vials for analysis.

Instrumental Analysis

The HPLC-MS/MS analysis was performed with an HP1090L HPLC (Hewlett-Packard Co., Palo Alto, CA, USA) connected in tandem to a TSQ-7000 triple quadrupole mass spectrometer (Finnigan MAT Instruments, San Jose, CA, USA) equipped with an atmospheric pressure ionization (API) interface. Separation was achieved on a 15 cm × 4.6 mm OD5 C_{18} column (Burdick and Jackson) with 5-μm particles, which was preceded inline by a 5-μm stainless steel frit to prolong the column lifetime. The solvent system was isocratic, consisting of 55:45 0.1% aqueous ammonium bicarbonate (pH 7.8):0.1% methanolic ammonium bicarbonate.

Negative atmospheric pressure chemical ionization MS/MS was achieved by using nitrogen as a sheath gas and argon as the collision gas. No API auxiliary gas was used. The pressure of nitrogen entering the API unit was kept constant at 40 psi (276 kPa). The argon gas pressure was 2×10^{-3} T (0.27 Pa). The API vaporizer and capillary temperatures were 400°C and 200°C, respectively. The discharge of the corona needle was 5 μA. The collision offset was at 24 V for optimal fragmentation. The electron multiplier voltage ranged from 1,800 to 2,400 V.

During an analysis, eight product ions were monitored in a multiple reaction monitoring (MRM) experiment at a scan time of 0.6 sec/ion. One quantification and one confirmation ion were monitored for each the native PNP and the $^{13}C_6$-PNP. In addition, one MS/MS transformation each was monitored for the PNP dimer and the $^{13}C_6$-PNP dimer and two MS/MS transformations for the mixed dimer. Table 1 lists the specific transformations monitored. The appropriate MRM specifications were recorded into an acquisition program initiated immediately upon injection of 10 μL of the sample extract by the HPLC autoinjector. The total analysis time per sample was about 3 min.

Typically, one analytical run consisted of 80 samples and was prepared for analysis by one analyst. Of these samples, 10% were QC materials, 10% were analytical standards, and 80% were unknown samples. One blank urine sample was analyzed per run. One instrument operated by one analyst was automated to analyze up to two analytical runs (160 samples) per day. We routinely analyzed 160 samples per day with one instrument and three to five analysts. Therefore, the total sample throughput was 1 sample per 18 min, or 30–55 samples per analyst day.

Data Processing/Analysis

Data were automatically processed by software supplied with the mass spectrometer. Each ion of interest was automatically selected, retention times were calculated, and the area was integrated. All data were checked for interferences, peak selection, and baseline determination and were corrected if found in error. The HPLC-MS/MS technique is highly selective such that an analyte is identified as PNP only if it meets the following criteria: *a*) elutes at a specified retention time; *b*) coelutes with the internal standard; *c*) possesses the same nominal mass as PNP; *d*) produces the same fragment ions as PNP; and *e*) has the same relative abundance (ratio of fragment ions) of fragment ions as PNP. Because of this specificity, interferences were rare. However, any interferences were easily recognizable because of a dramatic change in the ratio of the quantification ions to the confirmation ions of either the native analyte or the internal standard. When necessary, the data were reanalyzed to reflect these corrections.

The data were downloaded into an ASCII file and transferred from the UNIX-based operating system on the TSQ-7000 to a PC via an ethernet connection. The data were imported into an R:BASE (Microrim,

Redmond, WA, USA) database specifically designed for this analysis. The ratios of the quantification and confirmation ions were calculated. If the ratios were outside the 99% confidence limits, the analysis was repeated . If the ratios remained outside the 99% confidence limits in the second analysis, the data were considered unacceptable. All statistical analyses including QC evaluations were performed with SAS statistical software (SAS Institute, Inc., Cary, NC, USA).

Quantification

A calibration curve was constructed with the $\log_{10}$ of eight different analyte concentrations plotted against the $\log_{10}$ of the analyte response factor. The lowest standard concentration was near the method limit of detection (LOD) to ensure linearity at the low concentration end. The response factors (area/area internal standard) were calculated according to established methods (*24,26*). There were no apparent contributions of the isotope to the native ions and native to the isotope ion. A linear regression analysis of the calibration plot provided a slope and intercept from which concentrations of unknown samples could be calculated. Over the duration of this study, approximately 300 repeat determinations were performed for each point on the calibration curve.

Method validation. The analytical LOD of PNP was calculated as $3s_0$, where s_0 is the value of the standard deviation as the concentration approaches zero. The s_0 was determined as the *y*-intercept of a best-fit line of a plot of standard deviation of the standard samples used in the calibration curve versus the concentration (*27*).

Well-characterized pooled urine samples were enriched with a known amount of the native PNP standard and prepared according to the procedure outlined above. After the extraction, a known amount of $^{13}C_6$-PNP was added to each eluent, and the samples were analyzed. A control sample, which was a urine extract that did not contain endogenous PNP, was enriched with the same amount of native and labeled PNP. The recovery of PNP from the extraction was calculated as a percentage of the control PNP concentration, as the $^{13}C_6$-PNP corrected for any variation in instrumental response.

The method accuracy was evaluated by enriching urine samples with a known amount of native standard, preparing and analyzing the samples, then comparing the calculated concentration of the samples with the expected concentration. A linear regression analysis was performed on a plot of the calculated concentration versus the expected concentration. A slope of 1.0 is indicative of 100% accuracy.

Table 1. MS/MS specifications for MRM experiment.

Species	Precursor ion	Product ion	Neutral loss	Purpose of ion
PNP	138	108	NO	Quantification
PNP	138	92	NO_2	Confirmation
$^{13}C_6$-PNP	144	114	NO	Quantification
$^{13}C_6$-PNP	144	98	NO_2	Confirmation
2(PNP)	277	138	PNP	QA
PNP–$^{13}C_6$-PNP	283	138	$^{13}C_6$-PNP	QA
PNP–$^{13}C_6$-PNP	283	144	PNP	QA
2($^{13}C_6$-PNP)	289	144	$^{13}C_6$-PNP	QA

QA, monitored for quality assurance.

The method precision was determined by calculating the coefficient of variation (CV) of repeat measurements of the QC materials. At least 400 repeat measurements over a 2-year period were used in the calculations. In addition, both interday and intraday variation were determined.

Cross-Instrument Validation

To validate the use of additional TSQ-7000 mass spectrometers in our analyses, we analyzed at least 50 samples that had been previously run on a validated mass spectrometer. These samples consisted of standards, QC materials, and unknown samples. The data from these analyses were plotted against the previously obtained data. The slope of the resultant linear regression analysis had to be within ± 5% of 1.00 to cross-validate the new mass spectrometer.

Biological Monitoring of Methyl Parathion Exposure in Affected Residents

Concentrations of PNP were determined in the urine of people living in homes illegally contaminated with MP. State health officials collected urine specimens in 30-mL sterile Qorpak bottles (Qorpak, Pittsburgh, PA, USA). Specimen bottles were closed with polytetrafluoroethylene-lined screw caps, and the bottles were labeled with an appropriate specimen identification number. The identification number consisted of a five-character numeric code unique to the state and residence, followed by a three-character alphanumeric code that represented the particular individual tested and time of collection (i.e., A.M. or P.M.). These samples were shipped on dry ice overnight to our laboratory for testing.

Testing results were returned to the states within 48 hr, on average. These results were used to prioritize the relocation and decontamination efforts and to follow exposures over time to individuals in homes that were not decontaminated. The specific biologically driven action criteria for relocation and decontamination have been published elsewhere (28). Briefly, three action levels were defined (no further action needed, urine monitoring, relocation), and the urinary PNP concentrations required for these actions were broken into three categories (<1 year of age or pregnant, 1–15 years of age, ≥16 years of age). Urinary PNP concentrations resulting in continued monitoring ranged from 25 to 600 µg/L ppb, and those resulting in relocation ranged from 50 to 600 µg/L, both depending upon the age/pregnancy category.

Results and Discussion

Solvent extraction with dichloromethane proved to be simple and efficient for extracting PNP from the urine sample. The recovery of PNP at 5 and 10 µg/L was 85 ± 8% and 70 ± 3%, respectively. This simple extraction step, when performed in batches of 80 samples by one analyst, was completed in approximately 3 hr. Although the cleanup procedure was not highly selective, its simplicity allowed a high sample throughput for the method and minimized the errors normally associated with nonautomated, complex cleanup procedures.

Addition of an isotopically labeled standard ($^{13}C_6$-PNP) prior to sample manipulation, a technique known as isotope dilution (29), afforded us many advantages. Chemically, $^{13}C_6$-PNP behaves almost identically to PNP, but they are distinguishable

based upon the 6-atomic mass unit (amu) difference in their masses and respective fragment ions (Figure 2). For this reason, the ratio between their ions can internally correct for recovery of PNP in each individual sample. This eliminates the need for recovery surrogates. The automatic recovery correction reduces the error associated with the measurement and ultimately increases the method sensitivity.

As shown in the spectra in Figure 2, the major fragment ions produced during the MS/MS transformation correspond to the neutral loss of [NO] and [NO_2]. These losses, although common to most nitroaromatic compounds, provided a great deal of selectivity to our analysis. Because we observed some degree of dimer formation (i.e., 2PNP, $2^{13}C_6$-PNP, and PNP-$^{13}C_6$-PNP) during MS analysis, we also monitored the MS/MS transformations of the dimers to their respective monomers.

The method LOD was calculated to be 25 µg/L. Although this LOD was higher than previously published methods (23,24), it was suitable for categorizing samples into the various priority levels required for public health intervention. In subsequent studies we were able to lower the method LOD to 81 ng/L as calculated from calibration standards spiked into urine. We obtained over a 300-fold increase in sensitivity by modifying only a few mass spectral parameters. More specifically, by increasing the vaporizer and capillary temperatures by up to 50°C, the dimer formation became almost negligible. This increased the amount of the monomers available for the MS/MS transformations used in quantification and decreased the number of transformations monitored during the chromatographic retention window. These changes did not alter the calibration of the analytical system.

A calibration plot is shown in Figure 3. The PNP analysis was linear over two orders of magnitude, with an r^2 = 0.997. Similar calibration plots were obtained on multiple instruments, providing us flexibility and added resources when the sample load was
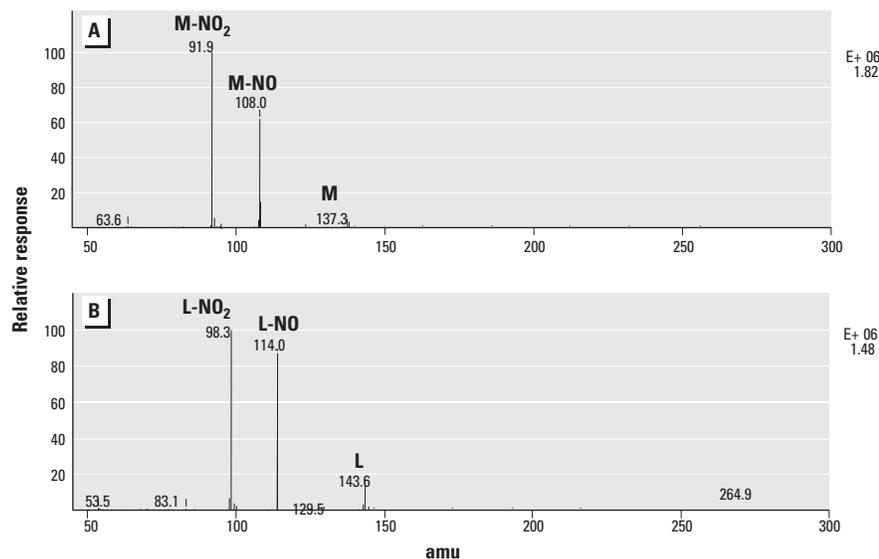
Figure 2. Both the native PNP (*A*) and the $^{13}C_6$-labeled PNP (*B*) fragment are almost identical but are distinguished based upon their 6-amu mass difference. The predominant neutral losses are NO and NO_2.
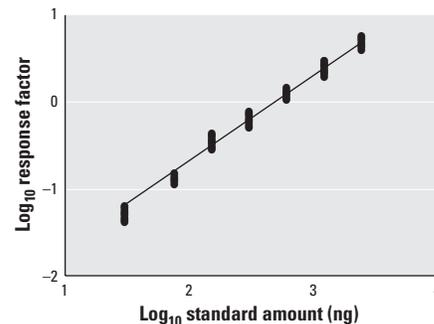
Figure 3. A calibration plot for PNP shows linearity over almost three orders of magnitude. The r^2 value is 0.999.

high. Cross-instrument validation studies among three additional mass spectrometers over the course of the study gave slopes ranging from 0.998 to 1.0. This indicates that less than 1% error was introduced by using multiple mass spectrometers.

The method's accuracy was indistinguishable from 100%. A linear regression analysis of a plot of the calculated concentrations of spiked samples versus the expected concentrations of the same samples yielded a slope of 1.0, which is indicative of a high degree of accuracy. On average, the calculated value was about 102% of the expected value.

A typical QC Shewart plot is shown in Figure 4. This plot includes samples analyzed on multiple instruments over a 2-year period and reflects both intraday and interday variation. The statistical validity of an analytical run was determined by evaluating the concentrations of the QC materials using the standard Westgard rules (*25*).

The overall CVs and the intraday and interday variations at two concentrations over the linear range of the method are shown in Table 2. As expected, the variation among days was a greater contributor to the overall CV than the variation within days.

The 1994 incident of illegal domestic use of MP by commercial applicators in Lorain County, Ohio, was our laboratory's first involvement in biomonitoring for MP exposure in exposed populations (*1,2*), and it affected approximately 250 homes. The decontamination efforts cost the government an estimated $20 million (*4,5*). From 1996 to 1997, similar incidences were reported in seven additional states: Mississippi, Alabama, Louisiana, Tennessee, Arkansas, Illinois, and Texas. A multiagency response team comprising the U.S. EPA, ATSDR, and state and local health officials was quickly constructed to respond to this public health crisis. Our laboratory assisted in prioritizing these decontamination efforts by measuring urinary PNP concentrations in about 16,000 samples from affected residents. To date, an estimated $75 million has been spent on relocation of
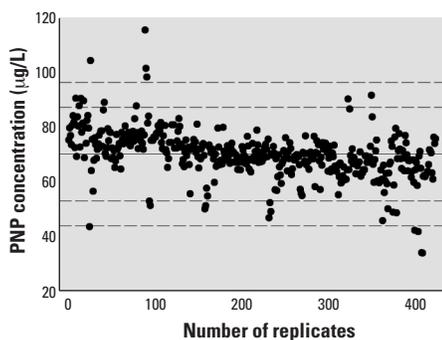
affected residents and decontamination and restoration of their homes (*5*).

A summary of the urinary PNP concentrations (micrograms per liter units) in about 16,000 samples is shown in Table 3. The creatinine-adjusted data for the same individuals are also shown. The mean concentration differed significantly from the median concentration for both the volume-based and creatinine-adjusted data, indicating that the data distribution was skewed. The mean PNP concentration was roughly 15-fold greater than the 95th percentile (upper limit) of the reference range (5.2 µg/L) (*30*). The median concentration was about 6 times the upper reference range; however, the median was well below the maximum concentration observed in the general U.S. population. The creatinine-adjusted mean was about an order of magnitude higher than the upper reference range (3.8 µg/g creatinine), whereas the creatinine-adjusted median was about 4 times higher.

Concentrations were detectable in about 57% of the samples measured. This detection frequency is slightly higher than that observed in the general U.S. population (41%) (*30*); however, the lower limit of reportable data for this study was 25 times higher than the LOD of the reference range study. If we were to impose the higher limit of detection on the general population data, <1% of the samples would have had detectable urinary PNP concentrations. Less than 1% of the samples contained interfering substances that precluded the report of their PNP concentrations.

PNP concentrations categorized by state and compared with incidental, occupational,

and poisoning concentrations are shown in Figure 5. The ranges shown by state include multiple discrete samples from the same individuals, both adults and children. The upper range of PNP concentrations in Illinois, Louisiana, Mississippi, and Tennessee were within the reported range of PNP levels encountered in occupational exposures. In addition, many of the samples tested in these states had PNP levels within the ranges seen in both nonfatal and fatal poisoning incidences. No fatalities were reported in any of the states tested.

Urinary PNP can result from exposure to parathion, MP, environmental PNP (as the degradation product of MP), and several other sources. Therefore, we verified that extremely elevated PNP concentrations (i.e., upper 1% of the distribution) in urine samples were, at least in part, a result of MP exposure by measuring the additional MP metabolites DMP and DMTP in the same urine samples. These metabolites were quantified using an isotope dilution GC–MS/MS method (*31*). There was a strong positive correlation between summed dialkylphosphate molar concentrations and PNP molar concentrations ($r = 0.78$, $p = 0.001$). When evaluated by the individual dialkylphosphate, both DMTP and DMP remained highly correlated with PNP concentrations ($r = 0.90$, $p = 0.0001$ and $r = 0.39$, $p = 0.007$, respectively). As seen in Figure 6, two extremely high values appear to skew the correlation of DMTP and PNP. However, when these two high values are removed, a good correlation
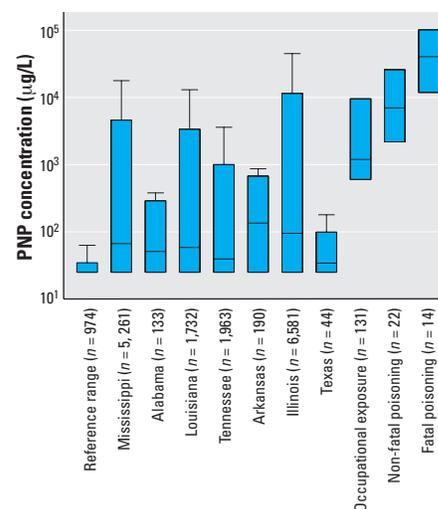
Table 2. Method variation over 2 years.

CV	Low pool (mean = 70 µg/L)	High pool (mean = 138 µg/L)
Total	12	14
Interday	9.2	9.4
Intraday	6.0	6.9

Table 3. Cumulative distribution of PNP in the urine of people who lived in residences illegally sprayed with MP.

Statistic	µg/L[a]	µg/g creatinine[b]
n	15,904	15,762
Mean	75	60
Geometric mean	33	27
Median	31	26
5th percentile	<25	5
95th percentile	230	190
Range	<25 to 45,000	0–21,000
Frequency of detection	57%	NA

NA, not applicable. [a]Nondetectable concentrations were given a value equal to LOD/√2 for statistical evaluations. [b]Nondetectable concentrations were corrected for creatinine using LOD/√2.

Figure 4. QC plot showing data accumulated over a 2-year period. Mean concentration is 70 µg/L with a CV of 12%.

Figure 5. Distribution of urinary PNP in residents of seven states compared with that of people exposed occupationally (*33–38*) or through poisonings (*33,36*). For individual states, bars indicate the 5th to 95th percentile, the error bar shows the maximum, and the line shows the mean concentration. For the occupationally and poisoning-exposed populations, the bars indicate the entire range and the line is the approximate mean.
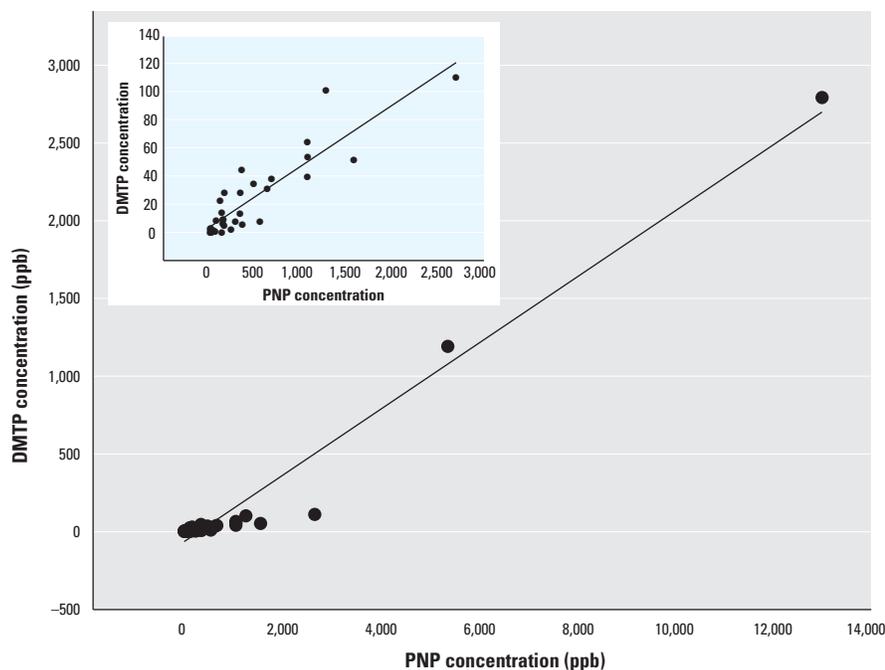
Figure 6. Correlation between PNP and DMTP was highly significant (Pearson coefficient = 0.90, $p = 0.0001$). Even when the two high values are removed (inset), the correlation remains strong (Pearson coefficient = 0.75, $p = 0.0001$).

(Pearson coefficient = 0.75, $p = 0.0001$) still exists (Figure 6 inset). In about 65% of the samples tested, DMP concentrations were greater than DMTP concentrations in the same sample. The DMP and DMTP concentrations ranged from less than the LOD to 145 μg/L and 460 μg/L, respectively. These data are consistent with the reported abundance of the dialkylphosphate metabolites of MP after occupational exposures (*32–34*). However, both DMP and DMTP concentrations in the samples tested had geometric means that were 11 and 5 times higher, respectively, than the geometric means of these same metabolites in the general population published in the Centers for Disease Control and Prevention National Report on Exposure to Environmental Chemicals (*35*). Because the dialkylphosphate metabolites are unlikely to be effectively absorbed through the skin because of their high polarity, these data suggest that the urinary PNP measured in these samples resulted, in part, from exposure to MP.

In many states, urine specimens were collected in both morning and evening. This sampling strategy was devised to evaluate whether a spot sample collected during the day would be representative of a 24-hr collection. Multivariate and paired bivariate analyses of the cumulative data and data categorized by state showed no significant difference between morning and evening PNP concentrations whether or not the data were creatinine adjusted. In fact, there was a moderate yet significant positive Pearson correlation ($r = 0.39$ volume-based, $r = 0.44$ creatinine adjusted, $p = 0.0001$) between cumulative morning and evening PNP concentrations for both volume-based and creatinine-adjusted analyses. In addition, some individual states showed strong correlations between morning and evening samples. These data suggest that similar PNP concentrations would be obtained regardless of the collection time.

In many states, residents whose urinary PNP concentrations placed them in the middle action category were monitored on a quarterly basis to assess any changes in exposure over time. A paired bivariate analysis showed a significant increase in urinary PNP concentrations after the first 3 months from a mean of 18 μg/g creatinine to a mean of 27 μg/g creatinine ($p = 0.0021$). For the next 6 months there was no significant change in urinary PNP concentrations ($p > 0.05$). However, after the next 3-month interval, the levels dropped significantly to 20 μg/g creatinine ($p = 0.0032$), nearer to the first-quarter values.

Conclusions

We have developed a sensitive and selective method for the quantification of PNP using isotope dilution HPLC–MS/MS. We applied this method to the analysis of about 16,000 samples collected from residents exposed to MP and/or PNP from their homes, which were contaminated by the illegal commercial application of MP. These data indicate that these residents had urinary PNP levels well in excess of those of the general U.S. population. These results enabled public health officials to implement a comprehensive, biologically driven response that included prioritization of relocation and decontamination efforts so that those who were most at risk were provided for most urgently.

REFERENCES AND NOTES

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