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Method for determination of acephate, methamidophos, omethoate, dimethoate, ethylenethiourea and propylenethiourea in human urine using high-performance liquid chromatography-atmospheric pressure chemical ionization tandem mass spectrometry

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Because of increasing concern about widespread use of insecticides and fungicides, we have developed a highly sensitive analytical method to quantify urine-specific urinary biomarkers of the organophosphorus pesticides acephate, methamidophos, omethoate, dimethoate, and two metabolites from the fungicides alkylenebis-(dithiocarbamate) family: ethylenethiourea and propylenethiourea. The general sample preparation included lyophilization of the urine samples followed by extraction with dichloromethane. The analytical separation was performed by high-performance liquid chromatography (HPLC), and detection by a triple quadrupole mass spectrometer with and atmospheric pressure chemical ionization source in positive ion mode using multiple reaction monitoring and tandem mass spectrometry (MS/MS) analysis. Two different Thermo-Finnigan (San Jose, CA, USA) triple quadrupole mass spectrometers, a TSQ 7000 and a TSQ Quantum Ultra, were used in these analyses; results are presented comparing the method specifications of these two instruments. Isotopically labeled internal standards were used for three of the analytes. The use of labeled internal standards in combination with HPLC-MS/MS provided a high degree of selectivity and precision. Repeated analysis of urine samples spiked with high, medium and low concentration of the analytes gave relative standard deviations of less than 18%. For all compounds the extraction efficiency ranged between 52% and 63%, relative recoveries were about 100%, and the limits of detection were in the range of 0.001–0.282 ng/ml.
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Introduction

Acephate (AP), methamidophos (MMP), omethoate (Omet) and dimethoate (Dmet) are organophosphorus (OP) pesticides. OP pesticides are the most commonly used insecticides in agriculture and the domestic field (Jaga and Dharmani, 2003; Kamanyire and Karalliedde, 2004). Their extensive and widespread use make it virtually impossible for anyone to completely avoid exposure. One feature of the OP pesticides that led to their wide usage is that they are much less persistent in the environment than other classes of insecticides. The mode of toxicity of this group has been firmly established as the inhibition of acetylcholinesterase, the enzyme responsible for catalyzing the breakdown of the neurotransmitter acetylcholine (Cocker et al., 2002; Kwong, 2002). The inhibition of the enzyme causes the accumulation of acetylcholine leading to symptoms related to the autonomous nervous system (abdominal cramps, nausea, diarrhea and salivation) and the central nervous system (dizziness, tremor, anxiety and confusion) (Mason et al., 2000; Delgado et al., 2004). Epidemiological studies have suggested that exposure to organophosphate pesticides can induce other chronic effects on the central peripheral nervous system, either after acute intoxication or as a result of lower level long-term exposure (Salvi et al., 2003; Miranda et al., 2004). Although the mode of toxicity is thought to be the same for all OPs, there are differences on OPs toxicity in humans (Eddleston et al., 2005).

The most widely used fungicides in agriculture are the alkali and metal salts of the alkelenebis-(dithiocarbamate) acids. The alkelenebis-(dithiocarbamates) can be divided into three subgroups, namely, dimethyl dithiocarbamates (ferbam, thiram and ziram), ethylene bisdithiocarbamates (EBDCs) (mancozeb, maneb, metiram, nabam and zineb) and propylene bisdithiocarbamates (propineb). Ethylene-

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thiourea (ETU) represents the main degradation product of the EBDCs and propylenethiourea (PTU) is the main degradation product of the propineb (Vettorazzi et al., 1995). They are foliar applied compounds that control many fungal diseases including early and late blights, leaf spots, rust mildew and scabs in various field crops such as fruits, nuts, cucurbits, vegetables, grapes and ornamentals. The occurrence of ETU and PTU as a contaminant, environmental decomposition product (Aprea et al., 1998; Van Lishaut and Schwack, 2000; Kontou et al., 2001), and urinary metabolite (Colosio et al., 2002; Debbarh and Moore, 2002; Sottani et al., 2003) is a cause for concern because of its mutagenic, teratogenic, carcinogenic and goitrogenic properties, described in experimental models (Khera, 1987; Doerge and Takazawa, 1990; Dearfield, 1994). Figure 1 shows the structures of the target analytes of our method.

Determination of metabolites in biological samples is the most practical and widely used method to estimate internal dose of OP pesticides resulting from various exposure pathways (Maroni et al., 2000; Aprea et al., 2002; Barr and Needham, 2002). Once human exposure occurs, OP insecticides are usually metabolized. The main metabolic reaction, common to most OP pesticides, is hydrolysis of the ester bond, with the production of alkylphosphate derivatives and chemical residues specific for each compound. As these metabolites are common to several OP pesticides, this indicator is not specific and is generally used to assess exposure to a group of parent OP compounds. In addition, specific metabolites of OPs can also be measured. The measurements of the alkylphosphate and the specific metabolite residues have both been used for biological monitoring.

Several methods have been reported for the measurement of the intact OP pesticides in environmental, agricultural and biological samples (Agüera et al., 2002; Barr et al., 2002; Lehotay, 2002). In fact, the majority of the intact OP pesticides are easily analyzed by gas chromatography (GC) with mass spectrometry (MS) detection (Anastassiades and Lehotay, 2003; De Niro et al., 2003; Wong et al., 2003; Rissato et al., 2005). However, AP, MMP, Omet and Dmet, due to their low molecular mass, polarity and /or thermolabile nature, are less GC amenable. Liquid chromatography with MS (LC/MS) is the obvious choice for the separation and detection of these analytes. Its applicability has been demonstrated in different matrixes including water, agricultural products (Ingelse et al., 2001; Perret et al., 2002; Mol et al., 2003) and biological samples (Aprea et al., 2002; Barr and Needham, 2002; Olsson et al., 2003). In addition, LC/MS is also the most reliable analytical method for the separation and detection of the metabolites ETU and PTU (Aprea et al., 2002; Barr and Needham, 2002; Colosio et al., 2002; Sottani et al., 2003; Blasco et al., 2004).

The extraction of AP, MMP, Omet, Dmet and the metabolites ETU and PTU is a challenge. These analytes are extremely water-soluble; some are not extractable using the more common SPE methods; or they require large amounts of organic solvents for extraction by liquid–liquid extraction (LLE). We propose a simple and efficient sample preparation method to extract ETU, PTU, AP, MMP, Omet and Dmet from a urine matrix. Our method employs lyophilization

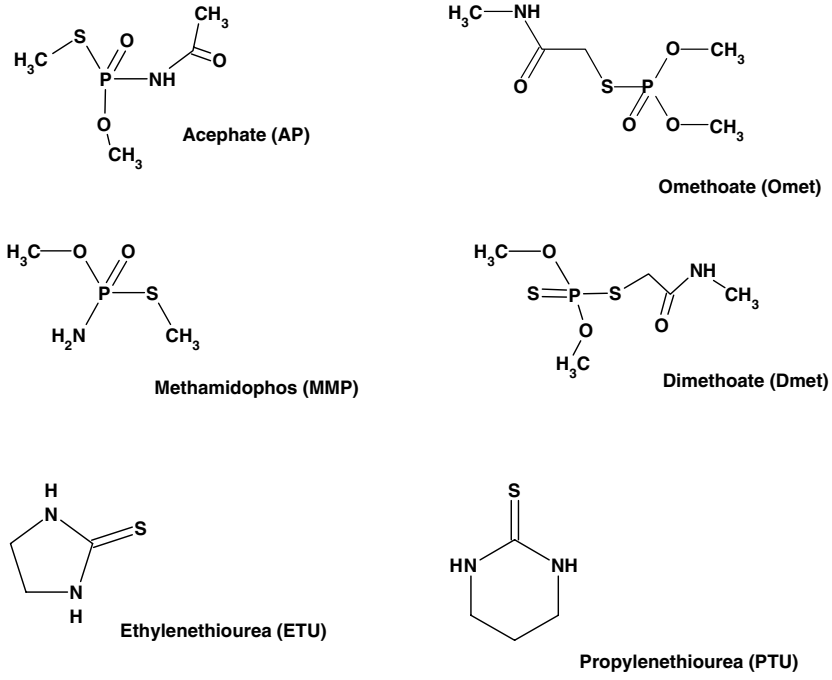

Figure 1. Structures of the target analytes.

and an extraction with dichloromethane followed by a highly selective and sensitive analysis using isotope dilution high-performance LC with atmospheric pressure chemical ionization MS (HPLC/APCI-MS/MS). We first developed and characterized this method using the Thermo-Finnigan TSQ 7000 triple quadrupole mass spectrometer. We later substituted a Thermo-Finnigan TSQ Quantum Ultra triple quadrupole for the TSQ 7000 in this method. Most results were comparable, although the sensitivity improved greatly. Thus, in addition, we present a comparison of sensitivity, precision, and other parameters for the two mass spectrometers.

Materials and methods

Chemicals

The native standards of AP (*O*,*S*-dimethyl acetylphosphoramidothioate), MMP (*O*,*S*-dimethyl phosphoramidothioate), Omet (*O*,*O*-dimethyl *S*-methylcarbamoylmethyl phosphorothioate), Dmet (*O*,*O*-dimethyl *S*-methylcarbamoylmethyl phosphorodithioate), ETU (imidazolidine-2-thione) and PTU (tetrahydropyrimidine-2(1H)-thione) were all purchased from Chemservice (West Chester, PA, USA). The labeled standards acephate-d_6 (AP-label), ethylene thiourea-ethylene-d_4 (ETU-label) and methamidophos-d_6 (MMP-label) were custom synthesized by Cambridge Isotope Laboratories (Andover, MA, USA).

All solvents used were of analytical grade. We obtained dichloromethane, acetonitrile and methanol from Tedia Company Inc (Fairfield, OH, USA). Formic acid was purchased from Fisher Scientific (Pittsburgh, PA, USA) and glacial acetic acid from JT Baker (Phillipsburg, NJ, USA). Deionized water was organically and biologically purified using a NANOpure Infinity ultrapure water system (Barnstead/Thermolyne, IA, USA).

Standard and Internal Standard Preparation

Individual stock solutions of the native AP, MMP, Dmet, Omet, ETU and PTU were prepared by weighing out 1 mg of each analyte and dissolving in 100 ml of acetonitrile. Stock solutions were stored at −70°C. Ten working standard solutions, each a mixture of an equal concentration of all the analytes, covering a range of 0.01–8.0 μg/ml, were prepared by diluting with acetonitrile appropriate volumes of the individual stock solutions in 100-ml volumetric flasks. The working standard solutions were stored at −20°C. Ten calibration standards were made by adding the working stock solutions to blank urine covering a range from 0.125 to 100 ng/ml. The calibration standards were made freshly before each analytical run.

The labeled internal standard stock solutions of AP-label, ETU-label and MMP-label were prepared by weighing approximately 1.0 mg of each isotopically labeled analyte into a 100-ml volumetric flask and dissolving with acetonitrile. These were stored −70°C. An internal standard working solution mixture of all the labeled analytes was prepared at 1.0 μg/ml in acetonitrile and stored at −20°C.

Quality Control (QC) Materials

Urine samples were collected from multiple (>30) donors, combined together, diluted with water (1:1 v/v) to reduce endogenous levels of the analytes of interest, and mixed overnight at 20°C. Our protocol for anonymous collection of urine was reviewed and approved by CDC's Institutional Review Board (IRB). The urine pool was pressure filtered with a 0.2-μm filter capsule and divided into four pools. The first pool (QCL), the second pool (QCM) and the third pool (QCH) were spiked with the native standard stock solution to yield concentrations of 0.5, 5.0 and 15 ng/ml, respectively. The fourth pool was not spiked. After being screened for possible endogenous analytes, the fourth pool was used as matrix material for calibration standards and blanks.

Sample Preparation

A 2-ml urine sample was pipetted into a 15-ml vial and spiked with 25 μl of the labeled internal standard working solution to give a urinary concentration of 12.5 ng/ml. The urine samples were vortex-mixed and placed into a −70°C freezer in custom-made Teflon racks for at least 4 h. After the samples were frozen, they were placed in a lyophilizer (Labconco, Kansas City, MO, USA). The lyophilizer was operated overnight in the automatic program mode without further manual manipulation. The program was set with the vacuum at 25.5 mT and the temperature at −34°C for 6 h following by 2 h at −20°C, 2 h at 0°C, 3 h at 20°C and, finally, 1 h at 22°C. The following day, after completion of the lyophilization process, dichloromethane (3 ml) was added to the residue in each sample tube to extract the analytes. The tubes were vortex-mixed for about 2 min. The samples were loaded onto 3 ml polyethylene cartridges with 20 μm pore frits (Varian, Walnut Creek, CA, USA); the breakthrough was collected into 15-ml centrifuge tubes. The extraction vials were rinsed with an additional 1 ml of dichloromethane, vortex-mixed and applied to the cartridges to combine with the previous breakthrough. The samples were concentrated to dryness using a Turbovap LV (Zymark, Hopkinton, MA, USA) at 40°C and 10 p.s.i. of nitrogen. Methanol (0.5 ml) was added to each tube, followed by vortex-mixing to rinse the tube. The samples were concentrated to dryness again. The residues were reconstituted with 50 μl of methanol and transferred to auto injection vials.

Chromatography and Mass Spectrometry Conditions

Chromatographic separation was performed using a Surveyor HPLC system (ThermoFinnigan, San Jose, CA, USA)

composed of an autosampler and HPLC pump. The column used was a Zorbax SB-C3, narrow-bore 4.6 × 150 mm, 5.0 μm (Agilent Technologies). The analytes were separated with gradient elution by using 0.1% formic acid in aqueous solution (solvent A) and 0.1% formic acid in methanol (solvent B). The initial mobile phase composition was 95% solvent A and 5% solvent B. The mobile phase was changed linearly over the next 5 min until the mobile phase composition was 40% solvent A and 60% solvent B. Over the next minute, the mobile phase composition was changed to 20% solvent A, and 80% solvent B then changed to 5% solvent A and 95% solvent B over the next minute. Finally, the mobile phase composition was allowed to return to the initial conditions and allowed to equilibrate for 2 min before the next injection. The total HPLC run time including equilibration was 9 min. The flow rate was 1000 μl/min and the injection volume was 5 μl. The divert valve was programmed to go to waste for the first 2 min and the last 2 minutes of the run. The Surveyor HPLC pump pressure was maximum 400 bar.

For the MS/MS analysis two different instruments were used: a TSQ 7000 triple quadrupole mass spectrometer and a TSQ Quantum high resolution triple quadrupole mass spectrometer (both from ThermoQuest, San Jose, CA, USA). The instruments were operated with an APCI source in positive ion mode with multiple reaction monitoring (MRM). For the TSQ 7000, the heated capillary and vaporizer temperatures were 250°C and 450°C, respectively. The corona current was set at 4 kV and the sheath gas pressure was 60 p.s.i. Collision-induced dissociation was performed using argon at 2.0 mT. The electron multiplier was set at 1400 V. The instrument parameters for the TSQ Quantum Ultra were as follows: vaporizer temperature 450°C, sheath gas pressure 45, auxiliary gas pressure 5 (arbitrary units), capillary temperature 350°C and collision gas 1.5 mTorr.

Extraction Efficiency

The extraction recovery of the method was determined at two concentrations, 10 and 50 ng/ml, by spiking six blank urine samples with the appropriate standard concentration and processing according to the method. Four additional blank urine samples (unspiked) were processed concurrently. Before the evaporation steps, all of the samples were spiked with a known amount of labeled internal standard to correct for instrument variation. The samples that were not spiked before preparation were then spiked with the appropriate native standard to serve as control samples representative of 100% recovery. After evaporating and reconstituting, the samples were analyzed. The recovery was calculated by comparing the responses of the blank urine samples spiked before lyophilization to the responses of the blank urine samples spiked after lyophilization

Quantification and Quality Control of Analytical Runs

Just before each analytical run, calibration standards were prepared by diluting the working standard stock solutions in blank urine. The concentrations of the 10 calibration standards ranged from 0.125 to 100 ng/ml for each of the analytes. To each run, 10 calibration samples, three quality control (QC) samples (QCL, QCM and QCH) and one blank urine sample were added; these were extracted and analyzed in parallel with the unknown samples. The area of the analyte divided by the area of the internal standard and plotted against the concentration of the sample to derive a calibration plot. The best-fit line of a linear regression analysis of the plot was used to derive an equation from which unknown sample concentrations could be calculated.

All QC pools were characterized before use to determine the mean and 99th and 95th control limits by consecutively analyzing at least 30 samples from each QC pool. QC samples were analyzed in runs with five replicates in six runs over 3 days. After establishing the control limits of the pools, individual QC samples contained within each analytical run were evaluated for validity using Westgard multirules (Westgard, 2002).

Limits of Detection

The limits of detection (LOD) were calculated for each analyte as three times the standard deviation of the noise at zero concentration ($3s_0$), where s_0 was estimated as the y intercept of a linear regression analysis of a plot of the standard deviation of the five lowest standards *versus* the expected concentration from six runs (Taylor, 1987).

Accuracy

The accuracy was calculated by spiking blank urine samples, at different concentrations and calculating the concentration using this method. A linear regression analysis was performed on a plot of the measured concentrations *versus* the expected concentrations. A slope of 1.00 was considered 100% accuracy.

Precision

The precision of the method was determined by calculating the relative standard deviation (RSD) of repeat measurements of the QC materials at three different concentrations (0.5, 5 and 15 ng/ml). At least 30 repeat measurements of QC materials were used to determine the method RSDs for each analyte.

Stability and adsorption of the analytes

The stability of the analytes in stock and working solutions at different temperatures were determined by monitoring changes in concentrations in samples stored at different temperatures in relation to a sample stored at -70°C (Baker

Table 1. The precursor and product ions, the collision energy and the relative retention time for the native analytes and their labeled internal standard.

Analyte	TSQ 7000		TSQ-Quantum		Relative retention time
	Precursor → product	Collision energy (V)	Precursor → product	Collision energy (V)	
ETU Q	103 → 44	−20	103 → 44	−26	2.59
ETU C	103 → 86	−25	103 → 86	−27	2.59
d_4-ETU	107 → 48	−20	107 → 48	−26	2.58
MMP Q	142 → 94	−19	142 → 94	−21	3.00
MMP C	174 → 94	−23	142 → 125	−18	3.00
d_6-MMP	148 → 97	−23	148 → 97	−20	2.98
PTU Q	117 → 58	−20	117 → 58	−21	3.18
PTU C	117 → 41	−28	117 → 60	−38	3.19
AP Q	184 → 49	−24	184 → 49	−27	3.78
AP C	184 → 143	−12	184 → 143	−15	3.78
d_6-AP	190 → 52	−24	190 → 149	−15	3.76
Omet Q	214 → 155	−20	214 → 155	−21	4.49
Omet C	214 → 125	−27	214 → 125	−28	4.49
Dmet Q	230 → 199	−14	230 → 125	−24	6.20
Dmet C	230 → 125	−28	230 → 171	−15	6.19

No labeled standard available for PTU, Omet and Dmet. PTU was quantified *versus* labeled [2]ETU, Omet and Dmet *versus* labeled AP.
Q, quaqntification ion.
C, confirmation ion.
Abbreviations: AP = acephate; Dmet = dimethoate; ETU = ethylenethiourea; MMP = methamidophos; PTU = propylenethiourea; Omet = omethoate.

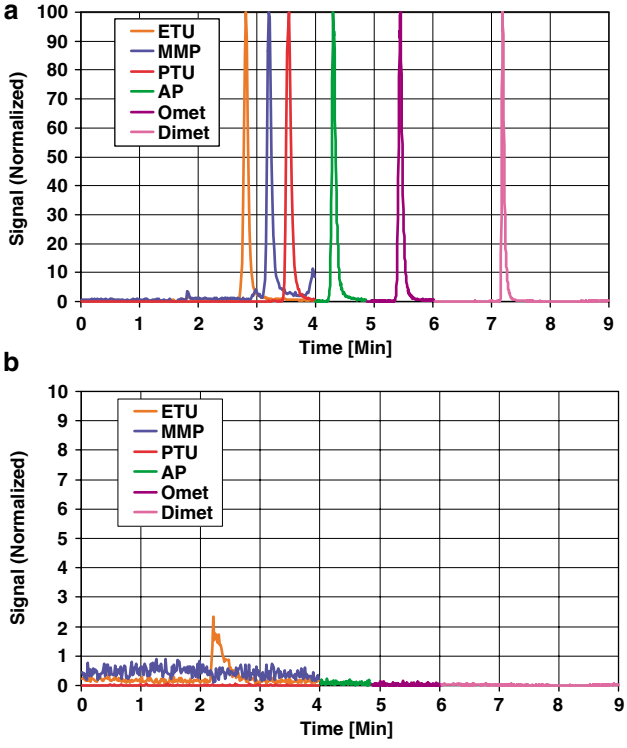

Figure 2. A typical reconstructed ion chromatogram of native analytes in spiked urine. 0.5 ng/ml (**a**) and a blank urine sample demonstrating no interferences (**b**).

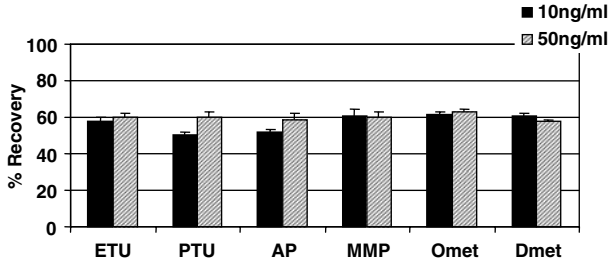

Figure 3. Percentage of recovery of the analytes in two concentrations of spiked urine after the standard cleanup procedure.

et al., 2005). The storage temperatures were −70°C, −20°C, 4°C, room temperature and 37°C. Samples were analyzed after 100 days of storage at each temperature. If any standard degraded over 10% during the storage time, it was considered unstable at that temperature.

To evaluate possible adsorption of the analytes onto glassware used in the method, 10 ml of a sample containing 50 ng/ml of each of the analytes dissolved in acetonitrile was placed in a silanized flask. Three different types of glassware used for sample storage or used during sample preparation were tested: large green cap glass vials (23 × 85 mm, BGC vials), small green cap glass vials (15 × 45 mm, SGC vials) and 15 ml disposable conical centrifuge tubes (C tubes) all from Kimble Inc. The sample in the silanized flask was considered the control at zero time adsorption. An aliquot of 1 ml (for each of the three types of glassware) was passed through 10 times. The test was performed in triplicate. The samples were analyzed to discover any changes in concentration.

For the analysis of the samples from the stability test and the adsorption test, a 25 μl volume of the sample was

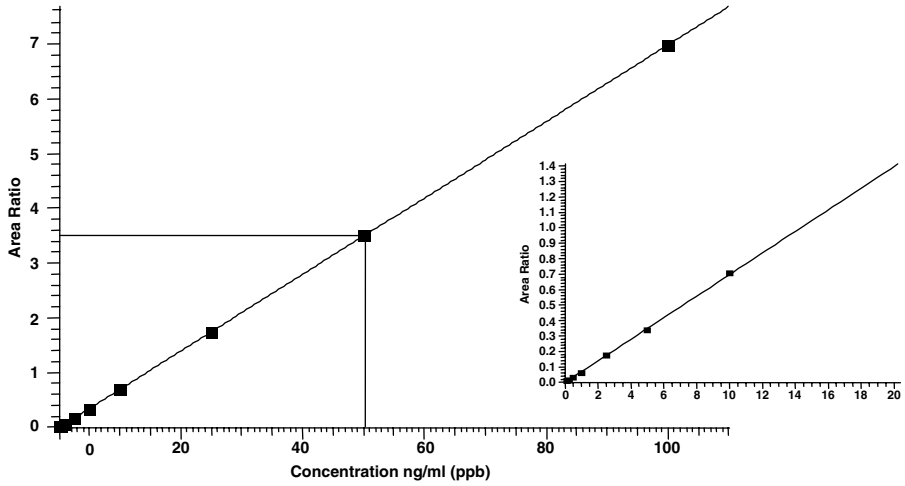

Figure 4. A calibration plot for ETU is shown. The correlation coefficient was 0.9999. This is a typical calibration plot for all analytes.

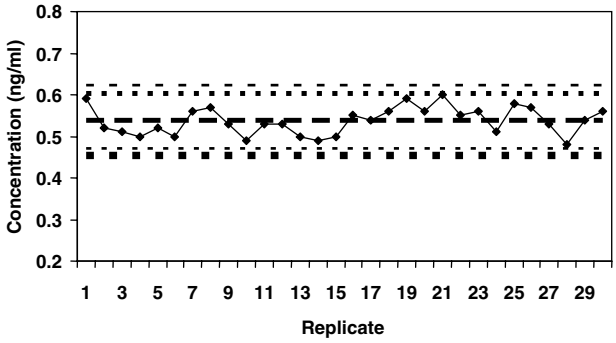

Figure 5. A Shewart plot for ETU in QC materials. The solid line represents the mean. The dashed lines represent the upper and lower 95th and 99th control limits.

combined with 25 μl of internal standard. The mixture was taken to dryness in a Turbo Vap LV (Zymark, Hopkinton, MA, USA) at 40°C and 10 p.s.i. of nitrogen. The residues were reconstituted with 50 μl of methanol and transferred to auto injection vials.

Matrix Effects

The matrix effects were evaluated by spiking three urine sample aliquots with different concentrations (1, 8 and 20 ng/ml), from each of seven different donors. Five replicates were analyzed from each urine sample aliquot. The replicates were divided in five analytical runs so that only one replicate from each aliquot was analyzed in each analytical run. A calibration plot with eight calibration standards was prepared and analyzed with each run. The urine samples were prepared for analysis according to the procedure already described above. An aliquot of each urine matrix was screened for possible endogenous analytes.

Biological Samples

Urine samples were collected as part of a study conducted by Center for Health Assessment of Mothers and Children of Salinas (CHAMACOS) at the University of California at Berkeley (Eskenazi et al., 1999). One part of this extensive project is to focus on pesticides exposures in Latin women and their children in the Salinas Valley in southern California. After collection the samples were frozen within 4 h and stored at -70°C before analysis. All protocols were reviewed and approved by a human subjects review committee and complied with all institutional guidelines for the protection of human subjects. About 499 urine samples from adults and children were analyzed using this method. The samples were divided in 20 runs; for each run we had a standard curve with eight calibration plots, ranging from 0.250 to 50 ng/ml, and three QC materials, each at a different concentration.

Results and discussion

The optimized precursor/product ion pairs as well as the collision offset energy for the target compounds on the two mass spectrometers, the TSQ 7000 and the TSQ Quantum Ultra, and the retention time, are summarized in Table 1. To improve selectivity of the analysis, we used the most abundant product ion as a quantification ion and the least abundant as a confirmation ion. All transitions were based on the $[M+H]^+$ precursor ions, except for the MMP confirmation ion on the TSQ 7000, which was based on a cluster ion with methanol $[M+H–32]^+$. Typical reconstructed ion chromatograms of a urine extract spiked with 0.5 ng/ml of the analytes and a blank sample are shown in Figure 2. The LC was optimized to achieve the best separation and retention for all analytes; it was performed under gradient conditions. The total run time was 9 min. The

Table 2. Summary of method specifications on TSQ 7000.

Analyte	LOD ng/ml (p.p.b.)	Standard curve R^2	Accuracy (%)	QC values			RSD		
				QCL	QCM	QCH	QCL	QCM	QCH
ETU	0.11	0.9994	100	0.54	5.11	15.90	10.39	6.53	6.99
PTU	0.12	0.9990	100	0.55	5.08	15.39	12.37	6.51	9.11
AP	0.28	0.9993	100	0.52	5.07	17.10	15.32	7.25	4.62
MMP	0.16	0.9949	100	0.46	4.97	14.74	9.39	9.69	11.84
Omet	0.05	0.9987	100	0.53	5.31	15.49	11.94	17.76	14.42
Dmet	0.03	0.9980	99.9	0.55	5.03	14.87	14.66	17.24	10.16

LOD: calculated as $3S_0$. Standard deviation at zero concentration (S_0) was estimated as the *y* intercept of a plot of the standard deviation of the five lowest calibration standards from six runs *versus* the expected concentration.
Standard curve: slope average of a linear regression analysis of 10 calibration standard from six runs. The data agreed nicely with the best-fit line with errors about the slope of less than 2%.
Accuracy: expressed as the percentage of the expected concentration that was quantified from six runs.
QC (quality vontrol) values: Average of QCL (low), QCM (medium) and QCH (high) from six runs. Blank urine pools were spiked with the native standard stock solution to yield a concentration of 0.5 ng/ml (QCL), 5.0 ng/ml (QCM) and 15.0 ng/ml (QCH).
RSD: relative standard deviation of the QC values from six runs.
Abbreviations: AP = acephate; Dmet = dimethoate; ETU = ethylenethiourea; LOD = limits of detection; MMP = methamidophos; PTU = propylenethiourea; Omet = omethoate.

Table 3. Summary of method specifications on TSQ-Quantum.

Analyte	LOD ng/ml (p.p.b.)	Standard curve R^2	Accuracy (%)	QC values			RSD		
				QCL	QCM	QCH	QCL	QCM	QCH
ETU	0.160	0.9999	100	0.51	5.36	13.37	7.86	4.90	3.47
PTU	0.004	0.9996	99.9	0.48	5.05	14.75	6.20	5.21	4.87
AP	0.023	0.9994	99.9	0.51	5.01	15.12	8.20	6.54	3.83
MMP	0.001	0.9995	99.9	0.50	5.26	15.26	4.49	4.72	2.51
Omet	0.025	0.9929	99.8	0.48	4.94	14.87	13.54	6.28	4.82
Dmet	0.004	0.9987	99.9	0.49	5.16	15.16	7.45	9.04	6.54

LOD: calculated as $3S_0$. Standard deviation at zero concentration (S_0) was estimated as the y-intercept of a plot of the standard deviation of the five lowest calibration standards from six runs versus the expected concentration.
Standard curve: slope average of a linear regression analysis of 10 calibration standard from six runs. The average error about the slope was less than 2%.
Accuracy: expressed as the percentage of the expected concentration that was quantified from 'six runs.
QC (quality control) values: average of QCL (low), QCM (medium) and QCH (high) from six runs. Blank urine pools were spiked with the native standard stock solution to yield a concentration of 0.5 ng/ml (QCL), 5.0 ng/ml (QCM) and 15.0 ng/ml (QCH).
RSD: relative standard deviation of the QC values from six runs.
Abbreviations: AP = acephate; Dmet = dimethoate; ETU = ethylenethiourea; LOD = limits of detection; MMP = methamidophos; PTU = propylenethiourea; Omet = omethoate.

divert valve of the HPLC system, which directs the solvent flow away from the mass spectrometer's ion source, was programmed to stay open for 2 min after injection to help reduce the chemical background and keep the ion source clean.

The measurement of ETU, PTU, AP, MMP, Omet and Dmet in urine is challenging because of their chemical and physical properties. These polar analytes are extremely difficult to extract from a polar urine matrix. A variety of methods reported in the literature have noted these difficulties; most methods use LLE requiring large volumes of organic solvents such as chloroform and dichloromethane (Colosio et al., 2002; Olsson et al., 2003; Sottani et al., 2003). The sample preparation that we propose is simple, efficient and reproducible. The lyopholization step has proven to be critical in enhancing recovery efficiency for these highly water-soluble compounds. Even though the lyophilization process takes approximately 14 h for 50 samples, it can be performed overnight and is an automated process. Lyophilization of the urine sample yields the driest possible sample, so that a small volume of organic solvent is required to extract these analytes. In our laboratory, the lyophilization technique has been applied with success for the measurement of other compounds including dialkyl phosphate metabolites of OP pesticides (Bravo et al., 2004).

Table 4. Stability and adsorption of the analytes.

Analytes	Stability (%)		−100 days incubation		Variation on adsorption (%)		
	−20°C	4°C	RT[a]	37°C	BGC vials	SGC vials	C tubes
ETU	100	100	100	100	1.2	0.8	0.9
PTU	100	100	100	100	0	1.5	0.3
AP	100	100	97	81	0	1.3	0.2
MMP	100	100	89	82	1.5	0.3	1.1
Omet	100	100	61	48	0.7	0.1	1.8
Dmet	100	100	68	49	1.4	1.2	1.6

[a]RT, room temperature.
At −70°C was considered 100% stability.
Percentage of variation on adsorption was calculated as 100−[(0 time adsorption/10 times adsorptions) × 100].
Abbreviations: Dmet = dimethoate; ETU = ethylenethiourea; LOD = limits of detection; MMP = methamidophos; PTU = propylenethiourea; Omet = omethoate.

Table 5. Matrix effects.

Analyte	Spiked matrix			Variation of matrix effect (%)		
	1 ng/ml	8 ng/ml	20 ng/ml	1 ng/ml	8 ng/ml	20 ng/ml
ETU	1.11 ± 0.14	8.44 ± 0.40	20.73 ± 0.63	9.1	5.3	3.5
PTU	1.10 ± 0.15	8.37 ± 0.37	20.53 ± 0.49	9.1	4.4	2.6
AP	1.07 ± 0.07	8.10 ± 0.30	20.17 ± 0.54	6.5	1.2	0.8
MMP	1.01 ± 0.05	8.19 ± 0.34	20.25 ± 0.61	1.0	2.3	1.2
Omet	1.07 ± 0.12	8.33 ± 0.36	20.41 ± 0.49	6.5	4.0	1.1
Dmet	1.06 ± 0.08	8.31 ± 0.47	20.22 ± 0.72	5.7	3.6	1.1

Urine samples from seven different individuals donors were spiked with the analytes and quantifying in five analytical runs. The percentage of variation was calculated as 100−[(Expected concentration/obtained concentration) × 100].
Abbreviations: AP = acephate; Dmet = dimethoate; ETU = ethylenethiourea; MP = methamidophos; PTU = propylenethiourea; Omet = omethoate.

The method's recovery efficiency ranged between 52% and 63% (Figure 3). Some analytes showed a small difference in recovery efficiency between the higher and the lower concentration. Using the isotope dilution technique, the individual recovery of each analyte in a sample is automatically corrected so variable extraction recoveries do not negatively affect the accuracy of the data obtained.

Figure 4 shows a calibration curve for ETU which is typical of the other analytes. The figure on the right shows the lowest standard points of the curve. Also a typical QC Shewart plot of ETU is shown in Figure 5. In this figure, we show the QC plot for QCL (0.5 ng/ml); this plot reflects both intraday and interday variation. The other analytes produced similar plots for all QC levels.

A summary of the method specifications is shown in Tables 2 and 3. The LODs of the method on the TSQ 7000 (Table 2) ranged from 0.030 to 0.282 ng/ml. For ETU and PTU, the LOD was 0.106 and 0.119 ng/ml; for AP, it was 0.282 ng/ml; for MMP, it was 0.162 ng/ml; and for Omet and Dmet, it was lower than 0.06 ng/ml. The LODs for most of the analytes (except for ETU) improve significantly when the TSQ-Quantum was substituted for the TSQ 7000. The LODs ranged from 0.001 to 0.160 ng/ml (Table 3). The LOD calculations were based on six runs of calibration curves. The signal intensities had higher magnitude in the TSQ Quantum Ultra under the same resolution conditions. Possible explanations for the better performance of the TSQ Quantum Ultra are new design features (orthogonal API source and hyperbolic quadrupoles) that have improved the sensitivity by reducing chemical noise, whereas increasing the signal. The LODs for both instruments are lower in magnitude than the LODs previously published for these analytes (Olsson et al., 2003; Sottani et al., 2003). The high sensitivity of this method makes it suitable for the measurement of internal doses resulting from incidental, low-level exposures such as those commonly occurring with environmental exposures.

For all analytes a slope average of a linear regression analysis of 10 calibration standards of six runs of calibration curves were calculated on the two instruments. The R^2 values were greater than 0.992 for all analytes. In addition, the method's accuracy was indistinguishable from 99.8% to 100%. The calculation was based on a slope average of linear regression analyses of plots of calculated concentrations of spiked samples *versus* the expected concentration of the same samples from six runs per instrument.

The method precision of each analyte, expressed as the RSD of repeated analyses of the QC materials, is also shown in Tables 2 and 3. The QC values were calculated as an average of six runs with five at each level in each run. In most instances, the RSDs were less than 18%.

We investigated the stability of the analytes and the possible adsorption of the analytes on the specific glassware used for storage or during sample preparation. Overall, the data suggested that the analytes are stable at −20°C and 4°C, and are not adsorbed on any of the glassware (Table 4). However, long-term (100 days) storage at room temperature or 37°C showed gradual deterioration for the OP pesticide (AP, MMP, Omet and Dmet), but not for the fungicide metabolites.

Possible matrix effects were investigated. Individual variation in pH and concentrations of salts and biomolecules in urine might affect the sensitivity of the method. Urine collected from different donors was spiked with three concentrations of the analytes. The variation of matrix effect on each concentration for each analyte was calculated, and the data are shown in Table 5. The variation of matrix effect ranged between 1% and 10% suggesting that individual differences in matrix composition did not significantly affect the performance of the method.

Table 6. Geometric mean (GM) concentration, range and percentage of detection frequency (DF) of ETU, PTU, AP, MMP, Omet and Dmet in 499 urine samples from the Salinas Valley.

Analytes	GM (ng/ml)	Range (ng/ml)	DF (%)
ETU	0.71	0.11–14.42	22.6
PTU	0.34	0.34–0.34	0.2
AP	1.43	0.28–46.89	3.1
MMP	1.97	1.01–9.33	1.3
Omet	1.28	0.32–11.64	0.6
Dmet	0.25	0.035–1.23	0.6

DF (%) = detection frequency (percent of sample with metabolite levels above the LOD).
Abbreviations: AP, acephate; Dmet = dimethoate; ETU = ethylenethiourea; MP = methamidophos; PTU = propylenethiourea; Omet = omethoate.

We applied this method with samples from pregnant women and their children in the Salinas Valley of California (Eskenazi et al., 2003). Table 6 shows the results from the analysis of 499 urine samples. The geometric mean of the concentrations, the range of the positive samples and the percentage of detection of the positive samples were calculated. The range of concentration was very narrow for most of the analytes in these samples. The highest percentage of detection was for ETU at 22.6% followed by AP at 3.1%.

The quality of the method has been tested and proven in practice by its use in routine analysis in studies involving exposed human populations. The QC system is robust providing consistent values over time showing that the QC materials and method are stable.

Conclusions

We present a high-throughput tool for measuring biomarkers of commonly used nonpersistent OP pesticides (AP, MMP, Omet and Dmet) and the fungicide metabolites (ETU and PTU) in human urine using isotope dilution HPLC-MS/MS. After performing the validation study and using the method for routine analysis, we conclude that the method is an accurate, sensitive and robust tool that can be applied to measuring these six biomarkers in environmental health studies to determine the health effects of the parent compounds.

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