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Quantitative determination of paraquat in meconium by sodium borohydride-nickel chloride chemical reduction and gas chromatography/mass spectrometry (GC/MS)

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Abstract

The objective of this study was to develop a procedure for the GC/MS assay of paraquat in meconium as a biomarker of fetal exposure to paraquat. The method involved a sodium borohydride-nickel chloride reduction procedure, liquid–liquid extraction of the perhydrogenated product, concentration, and GC/MS assay. The method demonstrated good overall recovery (102.56%) with %CV (inter-assay) of less than 13%, and a limit of detection of 0.0156 μg/g. Analysis of meconium samples from a study population in the Philippines ($n = 70$) showed a 2.8% prevalence of fetal exposure to paraquat.

Keywords: Paraquat; Meconium; Meconium analysis; GC/MS

1. Introduction

Paraquat (PQ) is a highly effective contact herbicide that is marketed worldwide as a fast-acting, non-selective compound for broadleaf weed control. As compared to most pesticides, PQ is extremely toxic to humans and the lack of strategies to manage PQ poisoning has resulted in high fatality rates [1]. In cases of advertent PQ ingestion among pregnant women, fetal deaths have occurred and levels of PQ in fetal blood were found to be four to six times higher than in maternal blood [2,3]. Newborn deaths have also been reported as a result of the pulmonary toxicant reaching the fetal lung tissues through blood circulation after birth, and eventually leading to rapid development of multiple organ failure [3].

While the effects of overt exposure to PQ on the fetus are evident, the immediate and long term effects of fetal exposure to environmental pollution levels of PQ or to subclinical doses of PQ are not known. In agricultural areas where PQ is used extensively, the herbicide is widely dispersed in the environment and has been found as residue in environmental, food, and biological samples [4,5]. Studies on prenatal exposure to neurotoxic pesticides have shown compelling evidence that these environmental contaminants can be transferred from the mother to the fetus or infant which can compromise brain development and function in later life [6–9].

Paraquat is a dopaminergic neurotoxin. Studies on the etiology of Parkinson's disease (PD) suggest that PQ may be an important environmental risk factor in the development of PD [10–13], due to the strong correlation between the incidence and development of PD and PQ exposure in certain human populations [10,12,14]. PQ is an efficient redox cycler that produces cation radicals in the presence of oxygen, resulting in cellular oxidative stress and eventually cell damage or death [15]. In animal studies, PQ has been reported to penetrate the blood brain barrier and specifically damage nigrostriatal dopaminergic neurons [11,16] which are crucial in maintaining normal motor function.

In animal models, studies on the effects of fetal exposure to PQ have shown that exposure to PQ during this critical period of rapid brain development can increase the risk of developing PD in later life [14,16]. In laboratory animals exposed to PQ *in-utero,* there was a significant reduction in the level of

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dopaminergic neurons in the brain [15]. As adults the animals were more susceptible to neuronal damage when re-exposed to PQ, and exhibited impairment of motor function [15–17]. Prenatal exposure to PQ has also been reported to affect cerebellar function in mice, disrupting the normal inhibitory and synaptic transmissions in the brain. This was found to affect motor coordination with the impairment becoming more apparent with advancing age [18]. Thus prenatal exposure to PQ in infants could potentially result in dopaminergic neuronal damage thereby increasing the risk in adult life of developing PD, as the number of neurons in the brain decreases with advancing age.

The fetal origin of PD has not been studied in humans due to the lack of an appropriate biomarker to detect fetal exposure to PQ [12,19]. The development of a method to measure a biomarker of exposure is needed for the study of subsequent adverse effects of antenatal PQ exposure. Likewise, the biomarker is important in identifying exposed infants early since therapies are emerging which can alleviate the oxidative stress and neuronal damage induced by PQ exposure [1,11,20].

Meconium is a repository of many compounds that are transplacentally transferred from the mother to the fetus. The compounds are metabolized by the fetus and are deposited in meconium as they are excreted in the bile or fetal urine. The latter occurs through fetal swallowing of amniotic fluid which to a major extent, consists of fetal urine. Meconium is formed as early as the 12th week of gestation and continues until birth. Thus, the deposition of xenobiotics in meconium is cumulative and provides a wide window to detect fetal exposure to many xenobiotics, including licit and illicit drugs, food additives, heavy metals, nicotine, alcohol, and recently, pesticides [21,22]. As compared to traditional matrices (i.e., urine, blood or saliva) in which measurement of pesticide levels is only indicative of transient or recent exposure, meconium analysis can detect exposure over the last two trimesters of pregnancy and can provide a cumulative measure of prenatal PQ exposure. It is therefore an ideal matrix for studying the potential relationship between prenatal PQ exposure and subsequent neurodevelopmental problems.

The objective of this study was to develop an analytical procedure for the extraction and quantitative determination of paraquat in meconium using gas chromatography/mass spectrometry. The GC/MS procedure was subsequently used to analyze meconium samples collected from infants born to mothers who reside close to a banana plantation in the Philippines, where spraying of paraquat is widely practiced.

2. Experimental

2.1. Materials

Paraquat dichloride tetrahydrate (1770 μg/mL in deionized water) and the internal standard, Dibenzyl [23] (98% purity), were obtained from ChemService (West Chester, PA, USA). Sodium borohydride (98% purity) was supplied by ACROS (New Jersey, USA) and Nickel chloride (technical grade) was ordered from Fisher Scientific (Fair Lawn, NJ, USA). Nitrogen (99.99% purity) and helium (99.999% purity) were ordered from Wilson Welding (Warren, MI, USA). Hexane and ethyl acetate (Fisher Scientific, Fair Lawn, NJ, USA) were of analytical grade and did not require purification. The sodium borohydride solution (40% (w/v)) was prepared by dissolving 4 g of the powder in 10 mL deionized water in a beaker. Nickel chloride (1% (w/v)) was prepared by dissolving 0.1 g of the crystals in 10 mL deionized water. Both reagents were prepared fresh before every analysis. A 2000 μg/mL stock solution of dibenzyl was prepared by dissolving 2 mg of the powder in 1 mL methanol. The stock solution was then serially diluted to 2 μg/mL.

2.2. Instrumentation

The assay was conducted using a Hewlett-Packard GC/MS system, composed of an HP 6890 gas chromatograph (GC) coupled with a 5973 Mass Selective Detector (MSD). Sample injections were made by an Agilent 7683 Automatic Liquid Sampler. Chromatographic separation utilized a 30 m DB5-MS J & W Scientific capillary column ([5%-phenyl]-methylpolysiloxane, 0.25 mm i.d., 1 μm film thickness) from Agilent (Wilmington, DE, USA). Instrument operation and data processing was done through the HP Chem Station (version B.01.100) software.

2.3. Meconium analysis

Meconium (0.5 g) was homogenized in 3 mL deionized water in a Sarstedt meconium processor tube (Sarstedt Inc., Newton, NC, USA). A 100 μL volume of dibenzyl (internal standard) at a concentration of 2 μg/mL (0.40 μg/g) was then added. The tubes were vortexed to mix, and then centrifuged at 4500 × *g* for 30 min. The supernatant was transferred into a 15 mL plastic screw capped test tube and centrifuged a second time at 4500 × *g* for another 30 min. The extract was then transferred into a 25 mL silanized screw capped test tube.

The chemical reduction of paraquat involved a sodium borohydride ($NaBH_4$) – nickel chloride ($NiCl_2$) reduction procedure adapted from Kanno et al. [24]. A 200 μL film of toluene was added to the surface of the liquid (antifoaming agent), followed by 400 μL of 1% (w/v) $NiCl_2$, and then 500 μL of 40% (w/v) $NaBH_4$. The $NaBH_4$ was added dropwise at first until the frothing subsided, then the rest of the volume was delivered. The tube was vortexed to mix the solution and the chemical reduction was allowed to proceed for 1.5 h at 15–20 °C using an ice-water bath. At the end of the chemical reduction step, 1 mL of 5 N sodium hydroxide was added to terminate the liberation of hydrogen.

The perhydrogenated PQ and dibenzyl were isolated from the chemical reduction mixture by liquid–liquid extraction. A 3 mL hexane-ethyl acetate (80:20 (v/v)) solution was added to the mixture and the tubes were capped tightly and tape-sealed. Liquid–liquid extraction was conducted using a Vibrax high speed rotary mixer (Fisher Scientific, Pittsburg, PA, USA) for 30 min and the layers were then separated by centrifugation at 4500 × *g* for 30 min.

The hexane-ethyl acetate layer was transferred into a silanized test tube and evaporated to approximately 200 μL

using a gentle stream of nitrogen at a room temperature of 25 °C. The test tube was vortexed and the extract was transferred into a silanized 1.5 mL high recovery vial (Agilent, Wilmington, DE, USA) where evaporation was completed to dryness. A 50 μL volume of hexane was pipetted into the vial and was then vortexed to reconstitute the sample prior to the GC/MS assay.

2.4. GC/MS assay

Chromatographic separation of the analytes involved the injection of 2 μL of the sample into the front inlet of the GC operating at 250 °C in the splitless mode for 0.75 min. Helium (carrier gas) flowed at a constant rate of 1 mL/min. The oven program started at 100 °C and was immediately ramped at a rate of 25 °C/min to a final temperature of 180 °C where it was held for 10 min. A post run of 5 min at 300 °C, involving a temperature ramp of 40 °C/min, concluded the oven program.

The MSD was interfaced with the GC at a temperature of 280 °C and was autotuned using perflurotributylamine (PFTBA, tuning standard). Ionization was achieved by electron impact using an emission current of 70 eV. The MSD was operated under selected ion monitoring (SIM) mode with a dwell time set at 40 ms.

Ions for the identification and quantitative determination of PQ were initially determined from the analysis of pure standards only (i.e. without meconium). Confirmation of the ions was done by the analysis of spiked meconium samples of known concentration in SCAN mode. Ions with significant abundance across decreasing concentrations were selected as either target or qualifier ions for the selected ion monitoring. The peak area (response) of the target ion chosen for PQ and dibenzyl were used to construct the calibration curve.

2.5. Quantitation and identification

The concentration of paraquat in meconium samples was calculated using a matrix-spiked calibration curve and the internal standard method [25]. Three spiked calibrators were prepared for each calibration level, using meconium previously determined to be negative for PQ. Dibenzyl was added to every sample at a concentration of 0.40 μg/g, and the samples were processed and assayed as described above. The concentration range for the calibration curve was from 0.0156 to 0.50 μg/g. The mean response of the target ion for PQ and dibenzyl were determined at each calibration level and the calibration curve was constructed by plotting the mean response ratio (response of analyte/response of internal standard) against the amount ratio (amount of analyte/amount of internal standard) [25]. The HPChem Station software uses both graphical and statistical techniques in generating the calibration curve. Curves (graphs) are generated by plotting the response ratio against the amount ratio using data from the analysis of spiked calibrators. With linear curves, a linear curve fit which uses a linear regression technique is used by the software to construct the curve, and a linear equation ($y = mx + b$) is generated for calculating the concentration of unknowns. In our case, we chose to use a quadratic fit because the resulting curve was parabolic. The curve fitting technique uses a least squares method to fit the detector's response and a quadratic equation ($y = ax^2 + bx + c$) was generated to calculate the concentration of unknowns.

Identification of the perhydrogenated PQ and dibenzyl was done by means of retention times and detection of target and qualifier ions. Identification was further ascertained using target to qualifier ion response ratios. The mean response ratios were calculated from target to qualifier ion response ratios obtained from the analysis of spiked calibrators and the limits were set at 20% of the mean response ratios [21,26,27]. The limit of detection (LOD) was established using the empirical approach [28]. The empirical LOD is the lowest concentration at which the target ion peak eluted at the expected retention time, all the qualifier ion peaks were detected, and where the target to qualifier ion response ratios were within the acceptable limits [21,27].

2.6. Quality control

Meconium samples that were negative for PQ were used for the positive and negative controls. Positive controls ($n = 3$) spiked at a concentration of 0.125 μg/g and a negative control were included in every batch of samples analyzed. Each run was evaluated in terms of % recovery, defined as the measured concentration divided by the spiked concentration and multiplied by 100, and the %Coefficient of Variability (%CV) (inter-assay). Batches in which the recovery was not within 80–120% and/or having a %CV greater than 10% were re-analyzed.

2.7. Human study

Meconium was collected from newborn infants in an ongoing clinical study of maternal/fetal exposure to pesticides conducted in Davao, Philippines. Samples were collected and pooled into sterile polypropylene containers (Phenix Research Products, Hayward, CA, USA) and stored at –20 °C. The samples were shipped to Wayne State University in dry ice for paraquat extraction and GC/MS analysis.

3. Results and discussion

3.1. GC/MS

Using the procedure described above, good chromatographic peaks were obtained using our established oven program on a DB5-MS capillary column. A good chromatographic separation, showing adequate baseline separation of PQ and dibenzyl was also obtained (Fig. 1).

The fragmentation pattern produced by electron impact mass spectrometry of perhydrogenated PQ is shown in Fig. 2 and is similar to that reported by Kanno et al. [24], showing the parent ion (*m/z* 196) and two predominant mass fragments (*m/z* 181, 96). The mass spectra of dibenzyl also showed a good fragmentation pattern with ions (*m/z*) 182, 96, and 65 as predominant mass fragments (Fig. 3). The high abundance of the molecular ion (*m/z* 182) indicated that dibenzyl has remained largely unchanged during the chemical reduction process. Despite this,

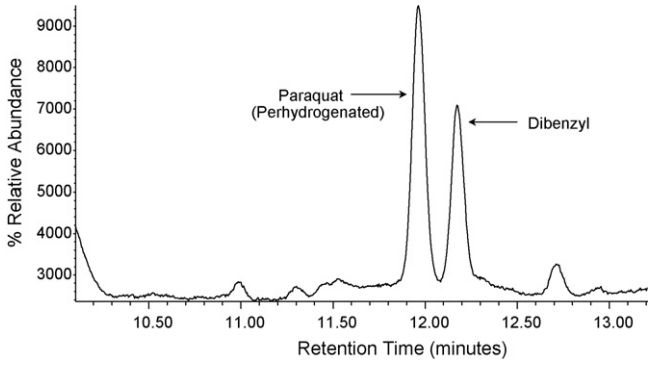

Fig. 1. Total ion chromatogram from the GC/MS assay of paraquat in spiked meconium (PQ: 0.25 μg/g; dibenzyl: 0.40 μg/g).

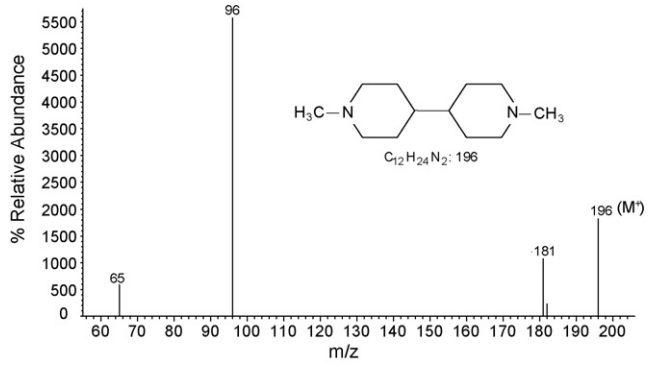

Fig. 2. Mass spectra and molecular structure of perhydrogenated paraquat (1,1′-dimethyl-4-4′-bipiperidine).

the response of the non-perhydrogenated molecule remained consistent at the spiking concentration on repeated injections, which made it an ideal internal standard for this method. The list of target and qualifier ions for PQ and dibenzyl are shown in Table 1.

The calibration curve for the quantitative determination of PQ was constructed using the peak area of mass fragment *m/z* 96 instead of the parent ion *m/z* 196. In this study, the response of ion 96 was found to be higher across decreasing concentrations as compared to the response of the parent ion. Use of *m/z* 96 as a quantitation ion (target ion) was also found to result in

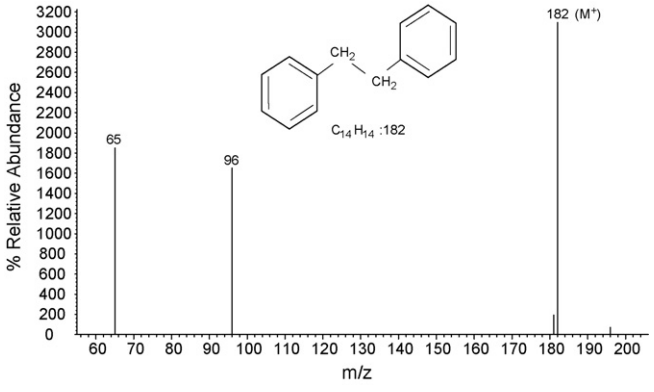

Fig. 3. Mass spectra and molecular structure of dibenzyl (1,2-diphenylethane) (non-perhydrogenated).

Table 1
Target and qualifier ions and retention times for the GC/MS assay of paraquat in meconium

Target compound	Target ion (*m/z*)	Qualifier ion(s) (*m/z*)	Retention time (min)
Paraquat	96	196, 181	11.96
Dibenzyl	182	96, 65	12.18

measured and spiked concentrations that were closer, especially in the lower concentration range. This was indicative of better accuracy as compared to values obtained from the calibration curve constructed from the response the molecular ion.

3.2. Method optimization

3.2.1. Extraction of paraquat in meconium

The aqueous extraction of paraquat from several matrices has been performed with good overall recoveries, normally by macerating the sample with deionized water [29,30]. We extracted PQ from meconium by homogenizing the matrix in deionized water using a Sarstedt meconium processing tube. The aqueous extract was free of suspended materials after double centrifugation, and facilitated the chemical reduction of PQ, as sodium borohydride readily releases hydrogen in water. This condition also promoted adequate separation of the immiscible phases during liquid–liquid extraction. The optimum responses of paraquat and dibenzyl were obtained after 15 min of aqueous extraction as compared to 10 or 30 min extraction times.

Attempts to isolate PQ from meconium by solid phase extraction (SPE) using C_{18} cartridges [4] resulted in a very low response of the perhydrogenated product. The procedure was also tedious and time consuming, which could potentially limit the number of samples that could be analyzed.

3.2.2. Chemical reduction and liquid–liquid extraction of perhydrogenated products

The chemical reduction of paraquat by the sodium borohydride-nickel chloride reduction system has been previously described [23,24]. The perhydrogenated product, 1,1′-dimethyl-4-4′-bipiperidine is soluble in organic solvents such as hexane, which could be easily evaporated and thus facilitate rapid concentration of the perhydrogenated PQ. The reduced compound is also amenable to gas chromatography in a non-polar DB5-MS stationary phase, which resulted in a good chromatographic peak. In the current study, the optimum response of the perhydrogenated product was determined by comparing the responses using different concentrations (20% versus 40% (w/v)) and volumes (400 μL versus 500 μL) of sodium borohydride, as well as different reduction times (60, 90, 180 min).

Liquid–liquid extraction was initially done using 100% hexane but the extract tended to gelatinize after centrifugation. A mixture of hexane and ethyl acetate was used to extract the perhydrogenated PQ and dibenzyl and to prevent gelation. Experiments involving different proportions of hexane and ethyl acetate (80:20 versus 90:10 (v/v)) for extraction were conducted,

Table 2
The effect of volume and concentration of $NaBH_4$ on the response of PQ at different spiking concentrations (chemical reduction time: 60 min; vol hexane: 3 mL; extraction time: 30 min)

Paraquat spiking concentration (μg/g)	400 μL 20% $NaBH_4$ mean (S.D.) response PQ	500 μl 20% $NaBH_4$ mean (S.D.) response PQ	500 μL 40% $NaBH_4$ mean (S.D.) response PQ
($N = 3$)			
2.0	357,776.7 (2791.9)	449,979.3 (21,040.0)	552,897.7 (2513.4)*
0.2	4,736.3 (85.0)	7,127.0 (690.5)	97,970.0 (2872.7)*
0.02	5,411.7 (196.8)	5,767.3 (593.3)	40,203.0 (1334.7)*
0.004	nd	nd	nd

Figures are means and standard deviation (S.D.). nd – not detected.
* $p < 0.05$ (one way ANOVA with post hoc Duncan analysis), 500 μL 40% $NaBH_4$ vs. 500 μl 20% $NaBH_4$ and 400 μL 20% $NaBH_4$.

Table 3
The effect of chemical reduction time and hexane:ethyl acetate ratio on the response of PQ at different spiking concentrations (500 μL 40% $NaBH_4$; vol hexane/ethyl acetate: 3 mL; extraction time: 30 min)

Paraquat spiking concentration (μg/g)	180 min reduction time 90:10 hexane/ethyl acetate mean (S.D.) response PQ	90 min reduction time 80:20 hexane/ethyl acetate mean (S.D.) response PQ	p^*
($N = 3$)			
0.500	397,741.7 (1336.0)	282,175.0 (2735.5)	<0.001
0.250	183,307.0 (1555.8)	147,632.7 (3385.4)	<0.001
0.125	67,105.0 (1069.1)	68,610.7 (1402.5)	0.213
0.062	6,653.3 (349.9)	30,651.3 (783.2)	<0.001
0.031	7,342.7 (245.7)	10,739.7 (1067.7)	<0.006
0.015	2,585.7 (502.0)	3,521.0 (404.0)	<0.07

Figures are means and standard deviation (S.D.).
* Comparison of means between 180 min reduction time and 90:10 hexane/ethyl acetate with 90 min reduction time and 80:20 hexane/ethyl acetate by Student "*t*" test with Bonferroni correction ($p < 0.008$).

and optimum responses of the perhydrogenated PQ and dibenzyl were obtained using an 80:20 hexane/ethyl acetate combination with no occurrence of gelation at an extraction time of 30 min.

A summary of the effects of selected analytical conditions on the response of PQ is shown in Tables 2 and 3. The 500 μL of 40% $NaBH_4$ was the optimum volume and strength of the reducing agent which yielded the maximum PQ response (Table 2). Optimum responses for PQ were also determined to be obtained using a 90 min reduction time and an 80:20 hexane/ethyl acetate combination instead of a longer reduction time (180 min) and higher hexane/ethyl acetate ratio (90:10) (Table 3). We adopted the 90 min reduction time and 80:20 hexane/ethyl acetate combination because of the higher responses that were detected at lower PQ concentrations, which is where we expect most of the PQ concentrations will be in clinical samples. Our processing time for chemical reduction was also reduced by half with this method.

3.3. Analytical performance

The method exhibited good overall recovery of PQ (102.56%) with %CV (inter-assay) of less than 13% (Table 4). Data from the analysis of spiked positive controls (mid-level concentration) averaged 96% with inter- and intra-assay variability (%CV) of 12.50 and 9.12%, respectively. The empirical LOD was determined to be 0.0156 μg/g. The calibration curve used for determining the concentration of PQ was non-linear (i.e. parabolic) and the use of a quadratic fit was found to enhance the accuracy of the calibration curve. Analysis of blank meconium samples showed no interfering peaks at the expected retention times of PQ and dibenzyl. In negative controls there were no co-eluting peaks detected for either analyte. The total run time for the assay is 18.20 min, which is relatively quick and suitable for analyzing a large number of samples. Table 4 shows the analytical performance of the method, which was gauged at high, medium and low spiking concentrations.

Table 4
Analytical performance of the method at three (high, medium, low) spiking concentrations

Spiking concentration (μg/g)	Mean (S.D.) calculated concentration (μg/g)	Mean recovery (%)	%CV (inter-assay)
($N = 3$)			
0.500	0.510 (0.023)	102.00	4.51
0.125	0.120 (0.015)	96.00	12.50
0.031	0.034 (0.001)	109.67	2.94
		Overall recovery (102.56)	

Calculated concentrations are given as means and standard deviation (S.D.).

3.4. Comparison with other methods

There are very few methods that employ the chemical reduction of paraquat for GC/MS assay and these methods have mostly analyzed biological fluids [4,31]. GC/MS analysis of PQ solely by sodium borohydride reduction has been reported by de Almeida for the analysis of human plasma and urine [31]. SPE procedures employed after chemical reduction resulted in recoveries ranging from 68–98% as compared to 102.56% overall recovery for our method, which employed direct chemical reduction of the meconium extract immediately followed by liquid–liquid extraction.

PQ is a positively charged, non-volatile, water soluble compound that is commonly analyzed using liquid chromatography (i.e. HPLC) after employing a sample clean-up procedure [4,31]. However, these methods lack the analytical specificity that can be provided by mass spectrometry, which the EPA recommends to confirm identity [4]. Although mass spectrometry coupled to liquid chromatography is now being utilized to enhance specificity in the analysis of biological fluids like whole blood [32] and urine [33], the pre-treatment, sample preparation and clean-up procedures prior to liquid chromatography still remain tedious and time consuming. Furthermore, the recovery of PQ from these reports using solid phase extraction techniques prior to ion-pair chromatography [32,33] range from 79.7 to 105.1%, which is comparable to the recovery obtained using our simpler methodology. In this report we describe a method that uses a chemical reduction technique for the GC/MS measurement and identification of PQ in meconium. The method is simpler than liquid chromatography techniques, has good sensitivity, and is capable of detecting concentrations in the ng/g level.

3.5. Analysis of paraquat in meconium

Analysis of 70 meconium samples from the study population showed two positive samples with concentrations of 0.106 and 0.046 μg/g corresponding to a 2.8% prevalence of fetal exposure to paraquat. This is the first study to report the detection of paraquat in meconium as a biomarker of fetal exposure to this environmental contaminant. This study also confirms the reported diffusion of PQ through the placenta [2,3], and also the possibility that PQ can exist as the parent compound in the fetus and is thus toxic to the fetal brain. Reported levels of PQ in certain solid biological matrices like human tissue [34], muscle, liver, lung and kidney [35] and rat brain [36] range from 0.015–10 μg/g. The concentration levels that we found in meconium are in the lower end of this range and may be reflective of the dose that comes from exposure to environmental pollution levels of PQ, as compared to intoxication cases that are commonly reported.

4. Conclusions

We have developed a method for the analysis of PQ in meconium involving a sodium borohydride-nickel chloride reduction procedure and GC/MS. The method has very good sensitivity and specificity and demonstrated good precision and accuracy. We were able to detect PQ in our analysis of meconium samples from the Philippines indicating fetal exposure to this environmental pollutant. The analysis of PQ in meconium as a biomarker of fetal exposure to PQ is important in the study of the potential adverse neurodevelopmental effects on the child and further into adulthood. It is also a critical instrument in the study of the potential relationship between fetal PQ exposure and the development of Parkinson's disease later in life.

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