Glyphosate and aminomethylphosphonic acid are not detectable in human milk

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ABSTRACT
Background: Although animal studies have shown that exposure to glyphosate (a commonly used herbicide) does not result in glyphosate bioaccumulation in tissues, to our knowledge there are no published data on whether it is detectable in human milk and therefore consumed by breastfed infants.

Objective: We sought to determine whether glyphosate and its metabolite aminomethylphosphonic acid (AMPA) could be detected in milk and urine produced by lactating women and, if so, to quantify typical consumption by breastfed infants.

Design: We collected milk (n = 41) and urine (n = 40) samples from healthy lactating women living in and around Moscow, Idaho and Pullman, Washington. Milk and urine samples were analyzed for glyphosate and AMPA with the use of highly sensitive liquid chromatography–tandem mass spectrometry methods validated for and optimized to each sample matrix.

Results: Our milk assay, which was sensitive down to 1 µg/L for both analytes, detected neither glyphosate nor AMPA in any milk sample. Mean ± SD glyphosate and AMPA concentrations in urine were 0.28 ± 0.38 and 0.30 ± 0.33 µg/L, respectively. Because of the complex nature of milk matrices, these samples required more dilution before analysis than did urine, thus decreasing the sensitivity of the assay in milk compared with urine. No difference was found in urine glyphosate and AMPA concentrations between subjects consuming organic compared with conventionally grown foods or between women living on or near a farm/ranch and those living in an urban or suburban nonfarming area.

Conclusions: Our data provide evidence that glyphosate and AMPA are not detectable in milk produced by women living in this region of the US Pacific Northwest. By extension, our results therefore suggest that dietary glyphosate exposure is not a health concern for breastfed infants. This study was registered at clinicaltrials.gov as NCT02670278.


Keywords: AMPA, glyphosate, human milk, lactation, organic food, aminomethylphosphonic acid, breastfeeding, environmental contaminants, pesticide

INTRODUCTION
Glyphosate (N-phosphonomethyl glycine), a widely used herbicide patented as a phytotoxicant in 1974 (1), functions by blocking the activity of 5-enolpyruvylshikimate-3-phosphate synthase (Enzyme Commission number 2.5.1.19), an enzyme required for the synthesis of tryptophan, phenylalanine, and tyrosine in plants and some microorganisms (2–5). Because these amino acids are not synthesized by humans, glyphosate would not be expected to have a physiological effect. Indeed, the human genome does not encode for 5-enolpyruvylshikimate-3-phosphate synthase, and a large body of epidemiologic and experimental literature supports the safety of glyphosate in mammals (5, 6). In addition, neither glyphosate nor its metabolite aminomethylphosphonic acid (AMPA)8 seem to bioaccumulate in animal tissues (7–9). In addition, most scientific evidence does not support contentions that glyphosate may cause cancer in humans, as recently concluded after a lengthy review by the European Food Standards Authority (10). The US Environmental Protection Agency (EPA) has authorized the use of glyphosate as a herbicide in noncrop and industrial areas since 1974 and in agriculture since 1976 (11). The safety of glyphosate use as an herbicide is periodically re-evaluated, with the last federal review completed in 1993 (12).

Despite its long-standing track record for safety, decades of research have resulted in a vast body of literature related to the clearance and disposition of ingested glyphosate. Studies in humans show that ~20% of diet-derived glyphosate is absorbed from the gastrointestinal tract, with the remaining ~80% excreted in the feces (13, 14), and studies conducted with rats suggest that nearly all absorbed glyphosate is rapidly excreted...
unchanged in urine (9, 15–18); little, if any, is metabolized to and excreted as AMPA. In fact, most AMPA in urine is thought to be the result of either the consumption of plants that have metabolized glyphosate into AMPA (12, 18, 19) and/or exposure to phosphonates found in detergents (20, 21).

Several studies have also investigated urine glyphosate concentrations of humans exposed to glyphosate via diet and other environmental sources (14, 22–25). These studies have consistently documented urine glyphosate concentrations of ~1–3 μg/L (in ppb), with the highest value being 233 μg/L (24). Curwin et al. (26) also reported urine glyphosate concentrations in 116 children living in “farm” and “nonfarm” households. Most samples (84%) had detectable concentrations with values similar to those reported in adults. There was no difference in urine glyphosate concentrations between children living in farm and nonfarm households (27). It is noteworthy that all measured urine glyphosate concentrations to date, even the highest, have not warranted a legitimate health concern based on the European Food Safety Authority’s allowable daily intakes and allowable operator exposure concentrations (14).

Of particular interest to our research group is the potential glyphosate exposure of infants during breastfeeding. Because there have been to our knowledge no studies published in peer-reviewed journals reporting glyphosate concentrations in human milk, this study (NCT02670278) was undertaken primarily to document typical glyphosate and AMPA concentrations in milk produced by lactating women living in the US Pacific Northwest—a highly productive agricultural region in which glyphosate-containing herbicides are routinely used (27). Maternal urine samples were also collected and analyzed. We hypothesized that concentrations of glyphosate and AMPA in milk and urine would be low, if even detectable. Important to testing this hypothesis was the use of newly optimized, matrix-specific assays with high sensitivities and specificities for the analytes (28).

METHODS

Human subjects

All procedures used in this study were approved by the Washington State University Institutional Review Board, and informed consent was obtained from each subject. A total of 41 healthy lactating women living in and around Pullman, Washington, and Moscow, Idaho, were included in the study, which was part of a larger investigation of international variation in human milk oligosaccharides and bacterial taxa as they relate to environmental exposure and sociocultural practices. To be eligible for participation, women had to be 1–3 mo postpartum, breastfeeding and/or pumping milk ≥5 times/d, and aged ≥18 y. Because we wanted to limit our subjects to healthy women who were nursing healthy infants, exclusion criteria included current breast infection, use of antibiotics in the previous 30 d, and having an infant with signs or symptoms of illness in the previous 7 d. Subjects completed a brief survey to document basic health and demographic variables, and body weight and height were measured at enrollment, which spanned from May 2014 through March 2015. All but 1 subject also completed a 5-question survey documenting potential glyphosate exposure from the environment and diet (Supplemental Figure 1).

Milk and urine collection and preservation

Milk and urine were collected between 0700 and 1100. After cleaning the breast (a step necessary to meet the needs of the larger, overarching project), ~30 mL milk was collected with the use of a Medela Symphony hospital-grade electric breast pump into a Medela Symphony single-use sterile collection container, immediately placed in ice, separated into aliquots while fresh, and then frozen at −20°C until analysis. A midstream urine sample was collected into a single-use sterile collection container. The sample container was immediately placed in ice, and urine was separated into aliquots and frozen at −20°C until analysis. One subject failed to provide a urine sample.

Glyphosate and AMPA analyses

Milk and urine samples were analyzed for glyphosate and AMPA at Monsanto with the use of liquid chromatography–tandem mass spectrometry methods optimized for and validated in each sample matrix (28). A Shimadzu Prominance 20A HPLC system coupled to an AB Sciex API 5500 triple-quadrupole mass spectrometer was used for analysis. Glyphosate and AMPA were quantitated with the use of multiple reaction monitoring. Two precursor–product ion transitions for each analyte and stable isotope labeled internal standard for each analyte were used to ensure the selectivity of the methods. Although 2 quantitative precursor–product ion transitions were monitored, the results were reported with the use of the most sensitive transition for each analyte. The assay was validated separately for milk and urine. Limits of detection (LODs) and quantification (LOQs) for glyphosate in milk were 1.0 and 10.0 μg/L, respectively; those for urine were 0.02 and 0.10 μg/L, respectively. LODs and LOQs for AMPA in milk were 1.0 and 10.0 μg/L, respectively; those for urine were 0.03 and 0.10 μg/L, respectively.

Glyphosate and AMPA concentrations in milk were independently confirmed by Covance with the use of the same liquid chromatography–tandem mass spectrometry method (28) with minor modifications, which included the use of an AB Sciex QTRap 5500 triple-quadrupole mass spectrometer. Because of differences in instrumentation, the LODs that used the more sensitive quantitative ion transitions were 6.0 and 9.0 μg/L for human milk glyphosate and AMPA, respectively, and the LOQ was 25.0 μg/L for both analytes.

It is noteworthy that duplicate aliquots (created from fresh milk at the time of collection) of each milk sample were sent directly, albeit separately, from Washington State University to Monsanto and Covance. Data generated by Covance were communicated directly to the principal investigators without prior disclosure to other coauthors.

Statistical analyses

All values for milk (n = 41) glyphosate and AMPA concentrations were below the LOD; thus, no statistical analyses on these data were warranted. For urine glyphosate and AMPA (n = 40), statistical analyses were conducted with the use of a generalized linear mixed model (SAS version 9.4; SAS Institute) assuming a Poisson distribution with a logarithmic link function. For concentrations less than the respective LOD values, one-half LOD (0.01 and 0.015 μg/L for glyphosate and AMPA, respectively) nominal values were used in the analyses. For
concentrations that fell between the LOD and LOQ, one-half LOQ (0.05 µg/L for both glyphosate and AMPA) nominal values were used in the analysis (29). All values presented represent means ± SDs.

RESULTS

Description of study population and glyphosate exposure

Basic demographic and anthropometric variables for the 41 study subjects are given in Table 1. Women were aged 29 ± 5 y, 67 ± 17 d postpartum, and had a BMI (kg/m²) of 26.8 ± 8.6. Most (75%) lived in an urban or suburban nonfarming region of the Palouse (a geographical area encompassing eastern Washington and northwestern Idaho), and most (58%) reported that they made no effort to eat foods characterized as organic, although they sometimes included them in their diets for convenience. Few subjects (15%) reported ever having personally mixed or used any type of weed killer; all but 1 of the women having reported ever doing so had mixed or used a weed killer containing glyphosate. In general, subjects were highly educated Caucasian women who participated in the study during either the summer or winter months.

Glyphosate and AMPA concentrations in milk

A summary of our findings concerning milk glyphosate and AMPA are found in Table 2. Regardless of whether the assays were conducted at Monsanto or Covance, none of the milk samples contained detectable amounts of either glyphosate or AMPA. As such, descriptive and statistical analyses were not warranted.

TABLE 1

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
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<tr>
<td>Variable</td>
<td>Value</td>
</tr>
<tr>
<td>Age, y</td>
<td>29 ± 5</td>
</tr>
<tr>
<td>Time postpartum, d</td>
<td>67 ± 17</td>
</tr>
<tr>
<td>Parity, n</td>
<td>1.8 ± 1.1</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>74.6 ± 24.2</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.8 ± 8.6</td>
</tr>
<tr>
<td>Lived on or near a farm/ranch, %</td>
<td>25</td>
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<tr>
<td>Strictly or mainly organic food choices, %</td>
<td>42</td>
</tr>
<tr>
<td>Had at some time personally used or mixed any type of weed killer, %</td>
<td>15</td>
</tr>
<tr>
<td>Highest attained educational level, %</td>
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<tr>
<td>High school</td>
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<tr>
<td>Undergraduate college degree</td>
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<tr>
<td>Ethnicity, %</td>
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<td>Caucasian</td>
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<tr>
<td>African American</td>
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<tr>
<td>Season of sample collection, %</td>
<td>12</td>
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<tr>
<td>Spring</td>
<td>46</td>
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<tr>
<td>Summer</td>
<td>17</td>
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<tr>
<td>Winter</td>
<td>27</td>
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Values are means ± SDs. AMPA, aminomethylphosphonic acid; LOD, limit of detection; LOQ, limit of quantification.

 Glyphosate and AMPA concentrations in urine

A summary of our findings concerning urine glyphosate and AMPA are found in Table 2 (raw data are available in Supplemental Table 1). Glyphosate was detectable in nearly all (n = 37) of the urine samples and was quantifiable in 29 of them. Glyphosate values ranged from below the LOD (<0.02 µg/L) to 1.93 µg/L, with a mean of 0.28 ± 0.38 µg/L. AMPA was also detectable in nearly all (n = 38) of the urine samples and quantifiable in 29 of them. Urine AMPA values ranged from below the LOD (<0.03 µg/L) to 1.33 µg/L, with a mean of 0.30 ± 0.33 µg/L. There were no significant effects of consuming organic compared with conventional foods or living on/near a farm compared with living in an urban/suburban region on concentrations of glyphosate in urine (P = 0.1870 and 0.8773, respectively) (Figure 1). Neither were there significant effects of consuming organic compared with conventional foods or living on/near a farm compared with living in an urban/suburban region on concentrations of AMPA in urine (P = 0.1414 and 0.2525, respectively) (Figure 2). Adjusting for potential covariates (age, time postpartum, BMI, parity) did not alter these conclusions. When raw, untransformed values were used in the analysis, there was a positive correlation (r = 0.57; P = 0.0001) between urinary glyphosate and AMPA concentrations. The strength of this association increased when log-transformed data were used (r = 0.68; P = 0.0001) (Figure 3).

DISCUSSION

The results herein provide evidence that the concentrations of glyphosate and AMPA in milk produced by healthy women are below the detection limits of available validated assays. In urine, glyphosate and AMPA were detectable in many samples, but concentrations were very low (<0.02 to 1.93 and <0.03 to 1.33 µg/L, respectively)—in fact, well below values reported in other

TABLE 2

<table>
<thead>
<tr>
<th>Variable Value</th>
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<tr>
<td>Milk (n = 41)</td>
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<tr>
<td>Glyphosate&lt;sub&gt;c&lt;/sub&gt; &lt;LOD</td>
</tr>
<tr>
<td>AMPA&lt;sub&gt;c&lt;/sub&gt; &lt;LOD</td>
</tr>
<tr>
<td>Urine (n = 40)</td>
</tr>
<tr>
<td>Glyphosate&lt;sub&gt;d&lt;/sub&gt; 0.28 ± 0.38</td>
</tr>
<tr>
<td>AMPA&lt;sub&gt;d&lt;/sub&gt; 0.30 ± 0.33</td>
</tr>
</tbody>
</table>

<sup>c</sup>Values are means ± SDs. AMPA, aminomethylphosphonic acid; LOD, limit of detection; LOQ, limit of quantification.

<sup>d</sup>LOD = 1 and 6 µg/L when milk was analyzed at Monsanto and Covance, respectively; glyphosate could not be detected in any of the milk samples analyzed.

<sup>e</sup>LOD = 1 and 9 µg/L when milk was analyzed at Monsanto and Covance, respectively; AMPA could not be detected in any of the milk samples analyzed.
healthy adult populations (<0.15 to 29 and <0.15 to 1.82 μg/L, respectively) (14, 24–26). To put these values in perspective, it is worth considering the EPA’s reference dose (RfD) value for glyphosate. The RfD is an estimate of the quantity of a chemical that a person could be exposed to every day for the rest of his or her life with no appreciable risk of adverse health effects (30). The RfD for glyphosate is 1.75 g $kg^{-1} \cdot d^{-1}$; this value is based on a “no-effect” concentration in animals (175 mg $kg^{-1} \cdot d^{-1}$) with a 100-fold safety factor (margin of exposure) (31). The EPA considers AMPA to be of similar or lesser toxicity than glyphosate and determined in 1994 that it should be exempt from regulation regardless of concentrations observed in food or feed (31). Thus, a 75-kg woman (typical weight for our study’s participants) could consume as much as 131.25 mg glyphosate/d with no expected negative effects. If 20% of dietary glyphosate is absorbed (i.e., 20% bioavailability) (14) and 100% of absorbed glyphosate is excreted into urine, such an individual would be expected to excrete 26.25 mg/d (26,250 μg/d) glyphosate in her urine. In the current study, the highest reported urine glyphosate concentration was 1.93 μg/L. As such, even allowing for a relatively high urine output (3 L/d), the highest glyphosate excretion in our study would be 5.79 μg/d, a value >4500 times lower than that which would be expected if the hypothetical mother described previously had consumed the RfD for glyphosate. The inclusion of AMPA, assuming equivalent toxicity, results in the highest excretion in our study of 2.58 μg/L (7.74 μg/d assuming 3 L urine/d) glyphosate + AMPA, an exposure >3000 times below the RfD; this combined calculation may become important if the EPA reconsiders the safety of AMPA (31).

Applying similar parameters and logic, a 5-kg infant can consume up to 8.5 mg/d (8500 μg/d) glyphosate and be below the RfD of this compound. Assuming a mean milk intake of 0.7 L/d (32–34) and a milk glyphosate concentration of 1 μg/L (the LOD value), then the maximum daily consumption of glyphosate by this hypothetical infant would be 0.7 μg/d—a value <12,000 times that which is thought to signal any semblance of a health concern (31).

FIGURE 1 Estimated means and 95% CIs for urine glyphosate concentrations of typical self-reported dietary pattern types (A) ($n = 17$ and $n = 23$ organic and conventional, respectively) and primary residence types (B) ($n = 10$ and $n = 30$ on farm and nonfarm, respectively). Upper and lower reference lines (dashed) represent LOQ and LOD values, respectively. LOD, limit of detection; LOQ, limit of quantification.

FIGURE 2 Estimated means and 95% CIs for urinary AMPA concentrations as they are related to typical self-reported dietary pattern types (A) ($n = 17$ and $n = 23$ organic and conventional, respectively) and primary residence types (B) ($n = 10$ and $n = 30$ on farm and nonfarm, respectively). Upper and lower reference lines (dashed) represent LOQ and LOD values, respectively. AMPA, aminomethylphosphonic acid; LOD, limit of detection; LOQ, limit of quantification.
The observed correlation between urine glyphosate and AMPA concentrations is also of interest. Our reanalysis of previously published data from Hoppe et al. (20) suggests correlations (Pearson correlations of 0.40 and 0.68 for raw and log-transformed data, respectively) very similar to our data. Because the strength of association was greater with the use of the log-transformed data, it is likely that this relation is not proportional but rather nonlinear in nature. Whether the AMPA was derived from endogenous metabolism of glyphosate, consumed as a component of the diet, or resulted from exposure to AMPA-containing detergents, however, cannot be determined from our study.

There are some limitations that should be taken into consideration when interpreting the results of this study. First, our subjects were relatively homogeneous in terms of anthropometrics, demographics, and geographical place of residence. Future studies should consider recruiting women of varied educational and ethnic backgrounds living in different regions of the United States. Second, it is noteworthy that the larger international study from which these samples originate was not designed to detect small differences in urine glyphosate and AMPA concentrations based on dietary choices, location of residence (e.g., urban compared with rural), or occupational glyphosate exposure. Nevertheless, we thought it was of topical interest to preliminarily explore these hypotheses given the availability of information. We note, however, that detecting such small-effect sizes at statistically significant concentrations and adequate statistical power would require 4–5 times as many observations than used in this study. Subsequent research on this topic should consider increasing sample sizes to the largest observations than used in this study. Subsequent research on this topic should consider increasing sample sizes to the largest possible extent while targeting enrollment of women who fit the hypotheses of interest, such as rarely or commonly consuming organic food, living on or off farms where glyphosate is used, and/or mixing and applying glyphosate as part of their livelihood. Studies might also consider investigating the possible effect of agricultural season on the outcomes of interest and the potential for breast infection (mastitis) to influence whether glyphosate and AMPA can be detected in a woman’s milk. Investigators are also urged to collect urine samples from exclusively breastfed infants to verify the lack of glyphosate and AMPA exposure during this important period of the life cycle and consider collecting complete breast expressions in case glyphosate and AMPA concentrations change during feeding.

Last, studying potential glyphosate and AMPA exposures from other sources (e.g., environmental and supplementary foods) before and after weaning might be of interest. However, it is important to note that glyphosate exposure would need to be much higher than those reported herein for maternal or infant exposures to become a health concern.

The authors’ responsibilities were as follows—MKM, MAM, DAG, and JLV: conceptualized and designed the study; MKM and DAG: designed the glyphosate exposure questionnaire; MKM: oversaw sample and data collection; JMC and KAL: collected the samples and administered the questionnaires; PKJ: oversaw the analysis of the samples at Monsanto; WJP and BS: carried out the statistical analysis; and all authors: read and approved the final manuscript. In 2014, MKM and MAM each received a $10,000 unrestricted research gift from Monsanto; these funds were used to support their research related to human and bovine lactation. These funds were neither needed for nor used to cover the costs associated with the project described in this article, because the milk was already being collected for another project funded by the National Science Foundation (1344288) related to international variation in human milk composition and because additional expenditures associated with the collection of urine samples were negligible. All costs associated with the chemical analysis of milk and urine samples at both Monsanto and Covance were paid for directly by Monsanto. MKM and MAM were once reimbursed for costs associated with economy travel and basic accommodations incurred for a trip they made to St. Louis, Missouri, to discuss study design and assay development with coauthors DAG, PKJ, and JLV at Monsanto. DAG, PKJ, and JLV are employees of Monsanto, which manufactures glyphosate. None of the other authors reported a conflict of interest related to the study.

REFERENCES


FIGURE 3 Correlation between urine glyphosate and AMPA concentrations. Vertical and horizontal axes represent the natural log scale (n = 40; 3 values hidden). AMPA, aminomethylphosphonic acid.


