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Detection of Glyphosate Residues in Animals and Humans

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Abstract

In the present study glyphosate residues were tested in urine and different organs of dairy cows as well as in urine of hares, rabbits and humans using ELISA and Gas Chromatography-Mass Spectroscopy (GC-MS). The correlation coefficients between ELISA and GC-MS were 0.96, 0.87, 0.97and 0.96 for cattle, human, and rabbit urine and organs, respectively. The recovery rate of glyphosate in spiked meat using ELISA was 91%. Glyphosate excretion in German dairy cows was significantly lower than Danish cows. Cows kept in genetically modified free area had significantly lower glyphosate concentrations in urine than conventional husbandry cows. Also glyphosate was detected in different organs of slaughtered cows as intestine, liver, muscles, spleen and kidney. Fattening rabbits showed significantly higher glyphosate residues in urine than hares. Moreover, glyphosate was significantly higher in urine of humans with conventional feeding. Furthermore, chronically ill humans showed significantly higher glyphosate residues in urine than healthy population. The presence of glyphosate residues in both humans and animals could haul the entire population towards numerous health hazards, studying the impact of glyphosate residues on health is warranted and the global regulations for the use of glyphosate may have to be re-evaluated.

Keywords: Glyphosate; Animals; Husbandry cows; Health hazards; Gas Chromatography Mass Spectroscopy (GC-MS); ELISA

Introduction

Glyphosate (N-phosphonomethyl glycine) is registered as herbicide for many food and non-food crops as well as non-crop areas where total vegetation control is desired. The predominating uses of glyphosate, in descending order, are stubble management, pre-sowing application and pre-harvest application (desiccation). Glyphosate is also used to prevent weeds in fields with glyphosate resistant genetically modified (GM) crops like soybean, rapeseed, corn, etc. Since 1996 the amount and the number of genetically engineered crops dramatically increased worldwide. It is estimated that 90% of the transgenic crops grown worldwide are glyphosate resistant [1]. The rapidly growing problem of glyphosate-resistant weeds is reflected in steady increases in the use of glyphosate on crops. Steams, leaves and beans of glyphosate resistant soy are contaminated with glyphosate. Moreover, due to the intensive use of glyphosate it was frequently detected in water, rain and air. Chang and coworkers [2] detected glyphosate concentrations in air and rain up to 2.5 µg/L in agricultural areas in Mississippi and Iowa. In Europe GM soybean for food and feed was admitted in 1996. All animals and humans eating this soy chronically incorporate unknown amounts of this herbicide. Residues of glyphosate in tissues and organs of food animals fed with GM feed (soybean, corn, etc.) are not considered or neglected in legislation. The influence of glyphosate residues on the quality of animal Products intended for human food is almost unknown. The incorporation of GM soybean meal in broiler feed significantly affects the color parameter for breast muscles [3]. In contrast Erickson and coworkers [4] did not find any effects on the performance and carcass characteristics of feedlot steers. Furthermore, glyphosate is a potent chelator fixing trace and macro elements [5-7].

The mode of action of glyphosate is through specific inhibition of 5-enolpyruvyl shikimate 3-phosphate synthase (EPSPS), an enzyme of the shikimate pathway that governs the synthesis of aromatic amino compounds in higher plants, algae, bacteria and fungi [8]. As this enzyme is absent in mammals it is often assumed that glyphosate is not harmful for mammalians. Even so, there is an ongoing debate about the safety of this herbicide. Firstly, long-term toxicology of the low glyphosate residues has not been investigated in vertebrates. Secondly although EPSPS is absent, glyphosate has been reported to inhibit other enzymes, e.g., enzymes of the cytochrome P450 (Cyp450) family [8]. Other inhibition pathways are reported. Richard et al. [9] reported that such as glyphosate inhibits Cyp450 aromatase inhibition, indicated crucial for sex steroid hormone synthesis.

Glyphosate also interferes with cytochrome P450 enzymes which include numerous proteins able to metabolize xenobiotics [10]. This may also act synergistically with disruption of the biosynthesis of aromatic amino acids by gut bacteria, as well as impairment in serum sulfate transport. Recently, it was suggested that gastrointestinal disorders, obesity, diabetes, heart disease, depression, autism, infertility, cancer and Alzheimer's disease are associated with Western diet [11]. Furthermore, genotoxic activity [12], teratogenic activity [13], and disturbance of the normal gut bacterial community [14,15] due to glyphosate are reported. Glyphosate showed cytotoxic effects on different cells *in vitro* [16-18], and Barbosa et al. [19], proposed that glyphosate may have contributed to the Parkinsonism due to its chemical similarity with glycine, a co-factor required for activation of the N-methyl-d-aspartase (NMDA) receptor, which controls excitatory actions in the central nervous system and is also involved in memory and learning. However, in clinical studies has not shown NMDA activity in relation to glyphosate poisoning [20].

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The aim of the present study was to investigate if glyphosate residues in different biological samples from humans and animals can be used to gain insight in the exposure situation.

Material and Methods

Samples

Samples from German dairy cows were collected as follow: urine from conventional husbandry (N=343), urine from cows kept in GM free areas (N=32), organs from slaughtered cows from conventional husbandry (gut wall [(N=32]), liver [N=41], kidney [N=26], lung [N=23] and muscles [N=6]. Urine samples also were collected from Danish cows (N=242). A total of 193 and 77 urine samples were collected from hares and fattening rabbits, respectively. In addition, a total of 99 and 41 urine samples were collected from humans with conventional or organic diet, respectively. Furthermore, a total of 102 and 199 urine samples were collected of healthy and chronically diseased humans. All samples were frozen at -20°C until analyzed.

Sample preparations

Tissue samples were minced to small pieces (~ 0.25 cm). In relation to the ability to retain water, samples were diluted with distilled water (Braun, Germany) at the rate of 1:1 (low water retention), 1:5 or 1:10 (high water retention). The specimens were heated at 100°C for 10 min, homogenized and frozen at -80°C for 8 h. Samples were carefully thawed at 40°C and centrifuged at 10.000 x g for 10 min. The supernatant was filtered with an ultra-centrifugal filter with a cut off of 3000 Da to remove proteins and peptides. Filtrates were centrifuged (10.000 x g) again at 20°C for 10 min and the supernatant was tested for glyphosate using ELISA and Gas Chromatography-Mass Spectroscopy (GC-MS). Urine samples were diluted with distilled water (Braun, Germany) at the rate of 1:20.

ELISA

Prepared samples were tested for glyphosate concentration by ELISA using glyphosate ELISA kits (Abraxis, USA) according to the manufacturer´s protocol. Test validation of ELISA was done in comparison with GC-MS and the Spearman rank order correlation analysis was calculated for human urine (N=14), cows urine (N=21), cow tissues (N=16), and rabbit urine (N=13). To study the recovery rate of glyphosate, meat samples were spiked with 100 µg of glyphosate which was carefully distributed in the meat and processed as mentioned above then the glyphosate was measured in the supernatant using ELISA.

Gas chromatography-mass spectroscopy

Glyphosate in urine and tissue samples was measured according to the procedure of Alferness and coworkers [21] with some modifications. Briefly, all chemicals used were of analytical grade unless stated otherwise. Urine samples and prepared tissue samples were thawed and equilibrated to room temperature. Samples were vortex mixed prior to transferring 100 µl aliquots to 10 ml screw-capped glass tubes containing 1 ml of acetonitrile. To each sample internal standard solution containing 13C215N-Glyphosate was added. After evaporation to dryness in a vacuum centrifuge, for derivatization 0.5 ml of 2,2,2-trifluoroethanol and cautiously 1 ml of freezing cold (-40°C) trifluoroacetic anhydride were added to the residue. The mixture was vortex mixed briefly and sonicated for 10 min and heated to 85°C for 1 h. After cooling the tube was uncapped and the solution was cautiously evaporated at 80-85°C without a stream of air or nitrogen. After cooling, the oily residue was dissolved in 200 µl of acetonitrile. The samples were measured using a GC-MSMS system. This system was composed of a gas chromatograph 7890 A equipped with a split/split less injector connected to 7000 Triple-Quad mass spectrometer operating in the chemical ionization (NCI) -Mode (both instruments from Agilent Technologies, Waldbronn, Germany).

Statistical analysis

The statistical analysis was carried out with GraphPad Prism 4 (GaphPad Software, La Jolla, USA). Two-way analysis of variance followed by unpaired Student t-test was used to identify significant differences between means.

Results

Validation of analytical method

The correlation coefficients between ELISA and GC-MSMS were 0.96, 0.87, 0.97, and 0.96 for cattle urines, human urines, rabbit urines and tissues, respectively, (Table 1). The recovery rate of glyphosate in spiked meat was 91% (Table 2).

Cattle

Glyphosate excretion in German dairy cows was significantly ($P<0.0001$) higher than Danish cows (Figure 1A). Surprisingly, cows kept in GM free region had significantly ($p<0.001$) lower glyphosate concentrations in their urine compared with cows under conventional husbandry (Figure 1B). Also glyphosate was detected in different organs of slaughtered cows including intestine, liver, muscles, spleen and kidney (Figure 1C). There were no significant differences of glyphosate residues in these organs.

Hares and rabbits

Hares showed significantly lower ($P<0.0001$) glyphosate residues in urine than fattening rabbit (Figure 2).

Humans

Glyphosate was significantly higher ($P<0.0002$) in humans feed conventional feed compared with predominantly organic feed humans (Figure 3). Also the glyphosate residues in urine were grouped

	Glyphosate (µg/ml or µg/g)						R^2
	ELISA			GC-MS			
	Minimum	Maximum	Mean ± SD	Minimum	Maximum	Mean ± SD	
Human urine (N=14)	0.1	71.3	9± 15	01	40	5.4± 11.5	0.87
Cows urine (N=21)	0.46	164	27± 42	0	164	35± 50	0.96
Rabbit urine (N=16)	2.37	70	17.9±19	3.17	42	12.5±12.1	0.97
Organs (N=13)	1.36	80	14.7± 21	4.7	108	20±26	0.96

Table 1: Spearman rank order correlation analysis between ELISA and GC-MS.

spiked glyphosate concentration (µg/g)	sample number	minimum (µg/g)	maximum (µg/g)	mean (µg/g)	median (µg/g)	standard deviation (µg/g)	recovery rate %
100	8	75.25	164.56	109.26	103.02	30.13	91

Table 2: Recovery rate of glyphosate in spiked meat ELISA.

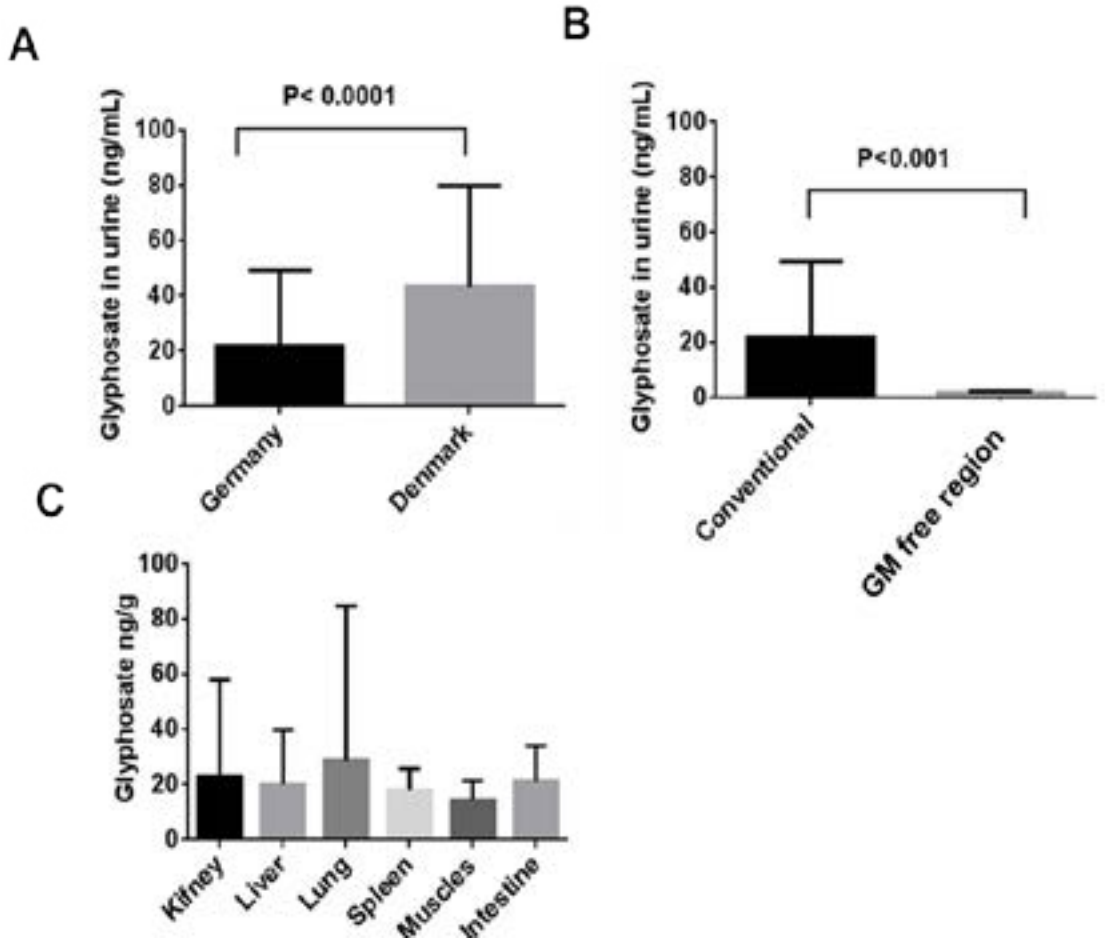

Figure 1: Glyphosate excretion in urine of cows. A) Comparison of glyphosate excretion in urine of German (343) and Danish (N=242) cows. B) Glyphosate in urine of conventional (N=343) and organic (N=32) livestock husbandry in Germany. C: Glyphosate accumulation in different organs.

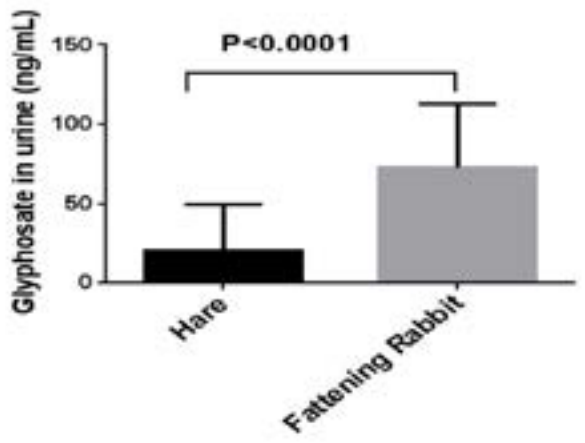

Figure 2: Glyphosate excretion in urine hares (N=193) and fattening rabbits (N=77).

according to the human health status. Chronically ill humans had significantly higher (P=0.03) glyphosate residues in urine than healthy humans (Figure 2).

Discussion

Glyphosate-containing herbicides are applied in large amounts to crops 2 to 3 times per season to remove weeds and dry out grain in a process called 'desiccation' [22]. Once applied, glyphosate accumulates in leaves, grains or fruit. Glyphosate residues cannot be removed by washing and they are not broken down by cooking [23]. Glyphosate residues can remain stable in foods for a year or more, even if the foods are frozen, dried or processed. Recently, many studies have proposed that glyphosate could impact the health of animals and humans. Despite glyphosate's global dominance as an herbicide, there is little testing of glyphosate residues in animals and humans. Previously, we recorded the presence of glyphosate residues in Danish dairy cows [7]. Glyphosate residues could differ from country to country (in some countries glyphosateis not regulated) and even within a country depending on the quantity, frequency and time of glyphosate application. The present study compared glyphosate residues in urine and different organs of

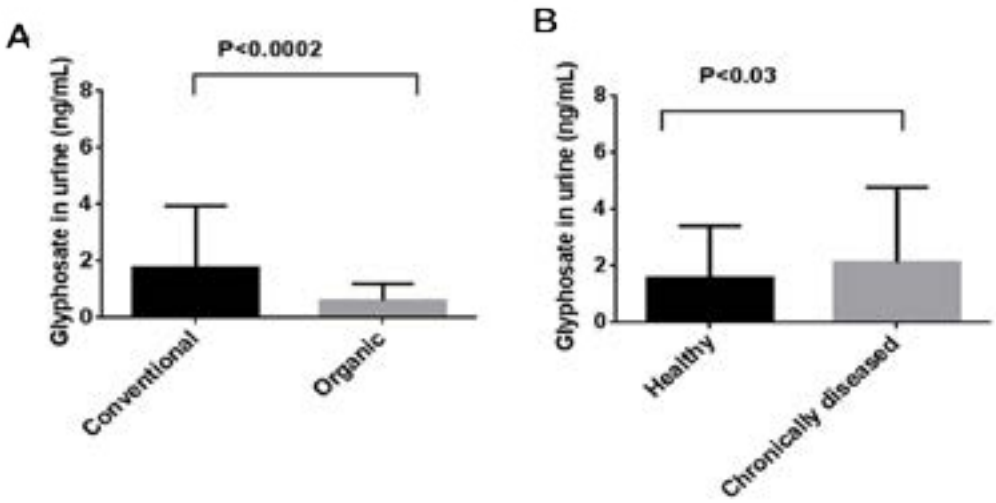

Figure 3: Glyphosate in humans. A) Comparison of glyphosate excretion with urine of humans with conventional (N=99) and predominantly organic (N=41) feeding. B) Glyphosate in healthy (N=102) and chronically (N=199) diseased humans.

German cows compared with Danish cows using ELISA and GC-MS. Other investigations were carried out to shed light on the presence of glyphosate in hares, rabbits and humans. The ELISA technique for these studies was validated by comparing paired samples with GC-MS (Tables 1 and 2).

Excreted glyphosate in urine of Danish cows was significantly higher than from German cows and represents a higher intake from feed. Interestingly, the glyphosate concentration in German dairy cows kept in a GM free region excreted much less glyphosate than conventionallymanaged cows. Thus, the prohibition of GM grains and soy prevent these animals from consuming glyphosate with their feed. Since organic farmers don`t live in an isolated area, the presence of low levels of glyphosate could be attributed to contamination by air and rain [2]. The presence of glyphosate residues in organs and meat of cattle is not a surprise since cows excretesignificant amounts of glyphosate in urine. An animal study with Sprague-Dawley rats reported that approximately 35-40% of the administered glyphosate dose was absorbed from the gastrointestinal tract so that urine and feces were equally important routes for elimination after one oral application [24]. These authors also found that glyphosate accumulated in bones. Considering the strong chelating stability of glyphosate for calcium, accumulation in bones is not surprising. Our own results showed that glyphosate is detectable in intestine, liver, muscle, spleen and kidney tissue.

Hares are among the first animals to enter fields after glyphosate is applied before sowing, in no-till cropping, and with pre harvest desiccation. Surprisingly,the many fold higher glyphosate excretion of domesticated rabbits than of hares (Figure 2) or Danish dairy cows was not anticipated, but probably reflects the high levels of glyphosate in their feed Tedesco et al. [25] evaluated the possible effects of GM soya bean on cell metabolism of rabbits. Although no differences in enzyme levels were detected in serum, a significant increase of lactic dehydrogenase, mainly the LDH1 iso-enzyme, was found especially in the kidney and heart.

In the present study, the median glyphosate concentration in urine (around 1 ppb) of people consuming predominantly organic food was significantly lower than in urine of people consuming conventional food. Thus, the prohibition of herbicide use in organic agriculture greatly reduces the intake of glyphosate. Glyphosate in urine of a generally healthy population was significantly lower than in urine from a chronically diseased population. Curwin et al. [26] mentioned that it is important to determine if glyphosate is consumed in conventional foods. Glyphosate in urine of humans (non-suicidal or accidental overdose cases) was measured in different populations. The presence of glyphosate in humans was previously reported [27] by monitoring 48 farmers, their spouses and 79 children (4-18 years) for glyphosate in urine the day before, as well as 1 and 3 days after glyphosate application. They reported detectable levels of glyphosate were found in urine on the day of application in sixty percent of the farmers (geometric mean was 3 ppb, the maximum value was 233 ppb, and the highest estimated systemic dose was 0.004 mg/kg). Farmers who did not use rubber gloves had five times more glyphosate in their urine. Mesnage et al. [28] detected 9.5 ppb glyphosate in urine of a farmer 7h after beginning pesticide handling. Moreover, the excretion of glyphosate in urine is not limited to farmers [26] and glyphosate has been described as a new environmental neurotoxin.

Exposure of mammals to glyphosate may cause loss of mitochondrial transmembrane potential and result in oxidative stress to liver and brain [29, 30]. Both apoptosis and autophagy are involved in glyphosate toxicity mechanisms [31] Case reports indicated that exposure to glyphosate was related to Parkinsonism [19, 32].

Conclusions

Glyphosate residue could reach humans and animals through feed and excreted in urine. Presence of glyphosate in urine and its accumulation in animal tissues is alarming even at low concentrations. Unknown impacts of glyphosate on human and animal health warrants further investigations of glyphosate residues in vertebrates and other non-target organisms.

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