Quantification of L-Abrine in Human and Rat Urine: A Biomarker for the Toxin Abrin

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Abstract

Abrin is a toxic protein found in the jequirity seed. L-Abrine (N-methyl-tryptophan) is also found in the jequirity seed and can be used as a biomarker for abrin exposure. Analysis of L-abrine was added to an existing method for quantifying ricinine as a marker for ricin exposure in human urine and analytically validated. Accuracy and reproducibility were enhanced by including a newly synthesized $^{13}C_1{}^2H_3$ -L-abrine internal standard. One-milliliter urine samples were processed using solid-phase extraction prior to a 6-min high-performance liquid chromatography separation. Protonated molecular ions were formed via electrospray ionization in a triple-quadrupole mass spectrometer and quantified via multiple reaction monitoring. Method validation included the characterization of two enriched urine pools, which were used as quality control materials. Endogenous levels of L-abrine were quantified in a reference range of 113 random urine samples at 0.72 ± 0.51 ng/mL. Urinary concentrations of L-abrine were monitored in an intentional rat exposure study for up to 48 h. Comparing the results from the human reference range and the animal exposure study indicates that this method is suitable for quantifying L-abrine within 24 h post-exposure. Quantification of L-abrine beyond 24 h is limited by rapid excretion of the biomarker and the level of the L-abrine dose.

Introduction

The phytotoxin abrin derives from *Abrus precatorius*, predominantly found in southeast Asia, but now also found in Florida and Central America (1–4). *Abrus precatorius* produces seeds known as the jequirity seed, rosary pea, and crab's eye. The scarlet variety (5) of *Abrus precatorius* produces a round seed which is about 100 mg (6), with a single black circular marking on a bright red shell. Due to their striking appearance, the jequirity seed has been used in the crafting of necklaces, belts, and ornaments (1,2,4,7,8).

Of primary concern is the wide availability of these seeds and their potential use as a source of poison. In the early 20th century, abrin was used to poison humans and cattle in several Asian countries by the use of sharp spikes fashioned from the dried paste of ground seeds (4,9,10). Pulverized seeds have also been used as a fish poison (7,10). Crushing or extracting the jequirity seed core releases the toxin abrin and biomarker L-abrine (11–13). The absolute quantities of abrin and L-abrine in the seed are about 0.12% (11,12) and 0.45% by mass, respectively (14). L-abrine has not been found in other plants and is considered to be a selective biomarker for exposure to *Abrus precatorius* and the toxin abrin (3).

At the cellular level, abrin inhibits protein synthesis leading to cell death (15–18). Abrin is very similar to the well-known toxin ricin because both poisons share the same mechanism of toxicity (6,19,20). Most documented cases of human abrin poisoning result from ingestion of the jequirity seeds. Toxicity to humans is largely dependent upon the integrity of the seed when swallowed. Younger seeds have a softer shell than more mature seeds (4) and are more likely to be toxic when ingested. If intact, the hard shell allows the seed to pass through the gastrointestinal tract without significant poisoning (4,8,15,21,22). Seeds that have been ground or masticated present a greatly enhanced poisoning threat when ingested (23). The abrin LD_{50} ranges from 10 to 1000 µg/kg (8,11,15), similar to the toxin ricin (LD_{50} = 100 to 1000 µg/kg) (7). Deaths occurred in Florida in 1949, 1958, and 1962 after children ingested one or more jequirity seeds (8,9). The onset of symptoms occurs within a few hours of ingestion and can last up to 10 days or longer (2,15,21). Symptoms of abrin poisoning include delayed severe gastroenteritis, followed by delirium, seizures, coma, and death (2). Death from abrin poisoning has been reported up to four days after the onset of symptoms and occurs as a result of persistent gastroenteritis (2,15,21).

Inhalation exposure experiments using rats have been conducted comparing the toxicity of abrin to ricin (24). The LCt_{50} values (inhalation vapor exposure lethal effects for 50% of population) for abrin and ricin were calculated to be 4.54 mg-min/m^3 and 4.54–5.96 mg-min/m^3, respectively. This equated to an inhalation LD_{50} values of 3.3 and 3.7 µg/kg for abrin and

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ricin, respectively. Survival time after inhalation exposure ranged from 30 to 66 h or 66 to 138 h depending on the dose level. Survival was inversely proportional to the dosage of the exposure. Postmortem examination of the animals indicated that damage was limited to the lungs, in contrast to systemic poisoning from parenteral injections.

Quantitative determination of abrin poisoning has previously been performed using a radioimmunoassay (25) in order to monitor abrin and ricin in the blood of cancer patients treated with these toxins. The method is sensitive to the 50–100 pg/mL range, with very little cross-reactivity noted for the two compounds. Alternative approaches have included the detection of anti-abrin antibodies (15) and the UV analysis of intestinal contents following poisoning (21).

Previous interest in the biomarker L-abrine results from a chemical structure and metabolism which are similar to tryptophan. Metabolic studies of L-abrine in cats involved the subcutaneous injection of 500 mg L-abrine and subsequent monitoring (26). L-Abrine was quantified in cat urine for up to 4 days after exposure with a total recovery of less than 20%. Excretion at a rate of 5–10% in the first 24 h, and about 2–3% in the following 24–48 h (26) supports the premise that some of the L-abrine survives metabolism and is detectable in human urine. Quantification of L-abrine has been performed via UV absorption of the compound at 235 nm and separation via thin-layer and high-performance liquid chromatography (HPLC) (3,27). Analysis of L-abrine and other N-methylamino acids has also been accomplished using reversed-phase HPLC (27).

This manuscript describes a new analytical method for the analysis of the abrin biomarker, L-abrine, in human urine. The ability to quantify biomarkers of toxins in urine provides a rapid, sensitive, and non-invasive approach for determining human exposure. In addition, measuring alternative biomarkers as described here reduces the safety issues associated with handling active toxins in the laboratory.

Experimental

Native L-abrine and ricinine were purchased from Sigma-Aldrich (St. Louis, MO) and Latoxan (Valence, France), respectively. Isotopically labeled L-abrine (methyl-$^{13}C_1{}^2H_3$) and ricinine ($^{13}C_6$), which were used as internal standards, were synthesized by Battelle (Columbus, OH) and Cambridge Isotope Laboratories (Cambridge, MA), respectively. The L-abrine and ricinine internal standards were produced with a purity of 98% and 99% respectively. All solutions were prepared and spiked with internal standard in 2-mL HPLC vials (National Scientific, Duluth, GA). Solutions were manipulated using Eppendorf (Westbury, NY) 10–100 µL and 100–1000 µL pipettors. The final HPLC analysis was performed using 300-µL autosampler vials (ARC, Wilmington, DE). Mixing of solutions was performed using a Fisher Scientific (Pittsburgh, PA) lab vortexor.

A urine pool was collected anonymously from 27 men and women at the Centers for Disease Control and Prevention according to an Institutional Review Board approved protocol. The urine pool was mixed overnight in a brown glass bottle using a magnetic stir bar and stirplate, filtered to 0.5 µm, and stored at –20°C in 25-mL aliquots. The urine pool was used as the matrix for blank solutions, calibrators, and quality control pools. Calibrators were spiked immediately prior to use, and quality control materials had been previously generated and required thawing from –20°C storage. Internal standard solutions were spiked into all solutions to a final concentration of 15 ng/mL, and the labeled ricinine was spiked to 1.0 ng/mL.

Three different stock aqueous spiking solutions, with accurately determined concentrations of about 8000, 80, and 8.0 ng/mL, were spiked into the urine pool on the day of analysis to create daily formulated standard calibrator solutions. Each standard calibrator contained both L-abrine and ricinine at slightly different concentrations because of weighing differences. The final concentrations of the L-abrine standards were 360, 230, 90, 45, 8.0, 4.5, 1.8, 0.68, 0.36, and 0.090 ng/mL. The final concentration of the ricinine standards were 320, 200, 80, 40, 7.1, 4.0, 1.6, 0.60, 0.32, and 0.080 ng/mL. All stock solutions, urine pools, standard calibrators, internal standard solutions, and quality control materials were stored at –20°C until needed.

Urine pools enriched at two concentrations served as the quality control pools. The composition of the low-concentration pool was 9.0 ng/mL L-abrine and 0.50 ng/mL ricinine. The high-concentration pool was 160 ng/mL L-abrine and 50 ng/mL ricinine. Quality control materials were mixed, aliquotted, and frozen until needed.

Synthesis of L-abrine internal standard

Commercially available tryptophan methyl ester was employed as the starting material, which was converted to the 2-nitrobenzenesulfonamide derivative (28). The derivative was then treated with labeled methyl iodide in dimethyl formamide methylating the amino group. Removal of the sulfonamide group with sodium thiophenylate followed by ester cleavage produced the desired L-abrine (see Figure 1). This labeled material had a nominal mass of 222 amu compared to the native material, which had a molecular weight of 218 amu. The internal standard was analyzed via the method described here; no isotopic interferences were noted between the native and labeled compounds.

Analytical procedure

The solid-phase extraction (SPE) procedure used here has been previously described as in our ricinine assay (29). In brief, the method utilized a 200-mg/6-mL polymeric SPE cartridge (StrataX, Phenomenex, Torrence, CA) to extract 1 mL of urine spiked with 100 µL of internal standard. HPLC-grade methanol, water and acetonitrile were used. The cartridge was conditioned with 6 mL of methanol followed by 6 mL of water. The 1.1-mL sample was loaded onto the SPE cartridge and then rinsed using 6 mL of 5% methanol in water. Acetonitrile (6 mL) eluted the retained compound.

The extracted sample was dried at 65°C using a Zymark TurboVap (Hopkington, MA) at 10 psig nitrogen for 30 min. Samples were dried in 15-mL conical glass test tubes (Kimble

Glass, Vineland, NJ). Reconstitution consisted of adding 200 µL of HPLC-grade water and vortexing for 10 sec using a Fisher Scientific minivortexer. The samples were then transferred to 300-µL HPLC vials for analysis.

An Agilent 1100 HPLC, equipped with G1312A HPLC pump, G1322A degasser, G1367A well-plate autosampler, and G1316A column heating compartment, was used for chromatographic separation. Ricinine and L-abrine were separated from other components using a 2 × 100-mm Polar RP Phenyl column (Phenomenex, Torrence, CA) with a 300-µL/min flow rate. The two HPLC mobile phases were (A) 10% methanol, 0.019% formic acid in water, and (B) 100% acetonitrile with 0.019% formic acid. Initial mobile phase conditions were 97% of (A) and 3% of (B). The gradient ramp to 50% (A) and (B) started at 0.1 min and ended at 2 min. The capacity ratio (k') for L-abrine and ricinine were 3 and 5, respectively. The autosampler injection loop was bypassed after a volume equal to 10 times the sample volume was flushed through the loop. The well plate sampler was preferable to the standard 100-vial autosampler because it allowed the use of a flush port for needle rinsing.

An Applied Biosystems (Foster City, CA) API 4000 triple-quadrupole mass spectrometer equipped with a turbo-ionspray source was interfaced to the HPLC. The instrument was operated in positive-ion multiple-reaction-monitoring mode. Transitions that were monitored for L-abrine were 219/188 (quantitation), 219/132 (confirmation) and 223/188 (internal standard). Transitions that were monitored for ricinine were 165/138 (quantitation), 165/82 (confirmation), and 171/144 (internal standard). Data analysis was performed using the Analyst 1.4.2 instrument software. A 1/*x*-weighted linear least squares regression was applied to the standard concentration versus the ratio of the areas of the quantification ion and internal standard ion. The response was linear over the entire range of standards with a correlation coefficient of 0.99 or greater. The area ratio of the quantitative ion to the confirmation ion, referred to as the confirmation ratio, was used to rule out interferences. The calibrators in each analytical batch were used to determine the average confirmation ratio to which unknowns were compared. An analytical batch consisted of 1 blank, 10 calibrators, 2 quality control (QC) materials, and any unknowns.

Method validation and stability of quality control materials

For method validation, 14 sets of 10 standards, blanks, and quality control samples were analyzed. The data from these experiments were used to characterize the QC pools (mean and coefficient of variation) and calculate the method limit of detection using the lowest three calibrators. The LOD was calculated by plotting the analyte concentration versus the absolute standard deviation of the three lowest standards (30). The *y*-intercept (s_o) was multiplied by three to calculate the method LOD. The LOD was calculated to be 0.09 ng/mL for L-abrine and 0.08 ng/mL for ricinine.

Both enriched QC pools were also used to determine analyte stability. QC samples were stored at room temperature for two weeks and then quantified. The same process was repeated for samples stored at –20°C for more than 12 months. No changes were noted in the room temperature samples or the –20°C samples.

Animal dosing experiments

Twenty male Wistar rats, approximately 6–7 weeks of age and weighing approximately 175–200 g upon receipt, were procured from Charles River Laboratories (Raleigh, NC). Maintained in polycarbonate cages with water bottles or with an automatic watering system in Association for Assessment and Accreditation of Laboratory Animal Care International-approved facilities, the animals were held in quarantine for 2 weeks prior to dosing. A total of 12 rats were dosed intramuscularly with L-abrine at 0.63, 3.13, and 5 times the expected abrin LD_{50} of 20 µg/kg. Although no toxin was used here, the concentration of the biomarker was increased by a factor of 4 to estimate an equivalent level of toxin, assuming that L-abrine is present in rosary peas at a concentration four times greater that the toxin abrin. A total of 12 rats were dosed intramuscularly with L-abrine: four at approximately 50 µg/kg body weight (bw), four at 250 µg/kg bw, and four at 400 µg/kg bw. Four control rats were dosed with water, which was the vehicle for all dosing experiments. Another four rats were dosed intraperitoneally with 20,000 µg/kg L-tryptophan to verify that endogenous L-tryptophan is not metabolized to L-abrine.

L-Abrine has little toxicity; no pain or distress was anticipated or observed in the treated rats. Humans have been given doses of L-abrine as high as 5 g (31) with no reported toxicity; assuming a 70-kg body weight, this equates to an L-abrine dose of 71 mg/kg bw. For an additional experiment with the

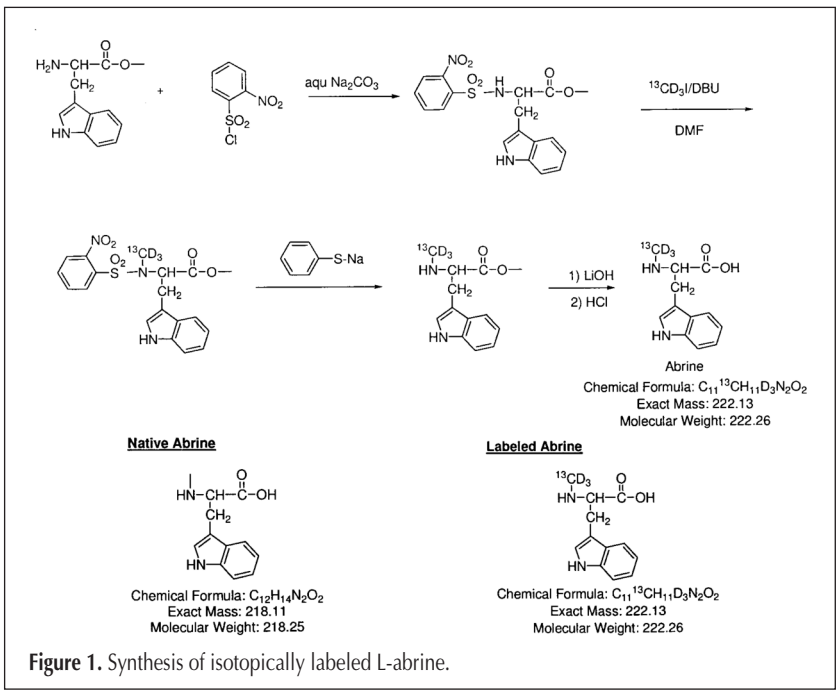


Figure 1. Synthesis of isotopically labeled L-abrine.

rats, a dose of 25 mg L-tryptophan/kg bw dose was selected to correspond to 25% of Huang's dose (31), a level 40 times higher than the highest L-abrine administered. Prior to dosing, body weight was measured and recorded. Urine was collected for approximately 24 h prior to dosing of L-abrine and 24 and 48 h after dosing. Urine samples were stored at –20°C. At 48 h after dosing, survivors were anesthetized with halothane.

Reference range of L-abrine and ricinine in the general population

A total of 113 urine samples, collected from random individuals with no known or suspected exposure to L-abrine or ricinine, were purchased from the Tennessee Blood Services (Memphis, TN). Donors of samples were anonymous, providing no personal identifiers beyond general demographics. Therefore, these samples were determined to be exempt from human subjects review. These were treated as unknown samples, spiked with 100 µL of internal standard, and extracted in the same manner as the blank, calibrators, and QC solutions. All positive results were quantified using water-based standards to generate the calibration curve.

Results and Discussion

We developed and validated a urinary biomarker method for abrin exposure by expanding on an existing analogous method for ricin (29) because both toxins produce similar symptoms in people (7). L-abrine was quantified in human urine using solid-phase extraction, liquid chromatography and isotope-dilution tandem mass spectrometry. Validation of the method included a limited animal study which was completed to monitor the kinetics and recovery of L-abrine in rats. A reference range of randomly collected urine samples was also examined to determine what concentrations may be detected people with no known exposure to abrin.

Sample cleanup and chromatography

The extraction of these biomarkers from urine utilized a polymeric sorbent bed which retained the analytes primarily through the interactions of unsaturated bonds (π-π interactions). L-Abrine and ricinine both possess cyclic unsaturated structures, although ricinine is not aromatic in nature. Since interaction of unsaturated bonds was the primary retention mechanism, adjustment of ionic strength and pH were not required for sample preparation. This was especially important in the case of L-abrine, which is a zwitterion, and ricinine is not pH active.

Once the analytes were retained on the cartridge, a rinse step using 5% methanol-in-water rinse was found to be effective in removing salts and other endogenous interferences. An acetonitrile elution was applied to efficiently break the π-π interactions. Other solvents that were considered included methyl tert-butyl ether for the rinse step, methylene chloride for the elution step, and higher concentrations of methanol and acetonitrile in the rinse and elution steps. Methylene chloride was shown to be a good alternative elution solvent for ricinine, but it did not elute L-abrine. No significant difference was noted between the use of neat methanol or acetonitrile for the elution steps.

Relative recoveries using the optimized extraction method were evaluated by spiking samples with the internal standard prior to and immediately following extraction. This was performed in duplicate, for a total of four samples, plus a blank sample (urine matrix plus internal standard only). The ratio of the pre-extraction spike was divided into the ratio of the post-extraction spike and multiplied by 100%. The relative recovery was measured to be 90% for L-abrine and ricinine. Because of the 90% recovery of the analytes from urine, the method has adequate sensitivity to detect low internal dose levels even with small sample volumes. The ability to quantify these biomarkers at a trace level is especially important considering the high toxicity of the toxins abrin and ricin. In addition, the two primary goals in the application of this method are to characterize patients with high-level exposures in order to provide effective medical treatment and to identify those with no significant exposure who may seek medical assistance because of concern that they were also exposed to these toxins. Trace level sensitivity is necessary to differentiate the worried-well from people with low-level exposure.

After the SPE clean-up step, the sample was further separated by reversed-phase liquid chromatography using a phenyl HPLC column. The acid functionality of L-abrine was protonated using dilute formic acid in the mobile phase, which produced longer retention times than when the molecule was in a zwitterionic state (deprotonated conditions). Alkaline conditions could not be used to neutralize the amine in L-abrine because of the stability of the column silica stationary phase. Ricinine is not pH active, so it was not retained differently in the column because of the addition of acid to the mobile phase. However, a suppression of ricinine was observed when the formic acid concentration of the mobile phase exceeded 0.019% formic acid.

The use of isotopically labeled internal standards improves method accuracy and reproducibility because the internal standard behaves the same chemically as the native biomarker while being differentiated by isotopic mass. Using isotopically labeled standards provides a more robust method which compensates for unexpected changes in sample extraction and chromatographic behavior. The isotopically labeled L-abrine was synthesized from tryptophan, and its synthetic route (see Figure 1) did not produce any unlabeled L-abrine as a by-product. This is especially important because any native material in the internal standard would produce a background interference that would require correction and limit the LOD. High concentrations of native L-abrine were also evaluated to ensure that there was no interference with the internal standard. Although not observed here, any interference of the native to labeled internal standard would produce a negative bias if not corrected.

Figure 2 illustrates the product ion spectra of the native and labeled L-abrine internal standard. The primary loss from the protonated molecular ion was the terminal N-methyl group. Unfortunately, the loss of the terminal N-methyl group caused the loss of the isotopically labeled component of the

internal standard. Although it is not ideal to lose the labeled component of the molecule during dissociation, the precursor ion still had a unique mass, 4 amu higher than the native compound. Thus, the transition of precursor-to-product ion was unique to the labeled L-abrine making it a suitable isotopically labeled internal standard.

The confirmation ratio is an important tool for eliminating any false positive results derived from endogenous interferences. In the tandem mass spectrometer, the confirmation ratio is the area ratio of the quantification and confirmation ions, usually the two most intense product ions from each respective native analyte. In the methodology presented here, the most intense product ion was designated as the quantification ion, and the second most intense product ion was designated as the confirmation ion. In each batch, which included a matrix blank, calibrators, and quality control materials, the average confirmation ratio was measured using the calibrators by dividing the confirmation-ion area into the quantification-ion area. Then the confirmation ratio was used, with a tolerance of 30%, to evaluate the quality control pools and any unknown sample results above the method limit of detection. Because of variation in the relative response of the quantification and confirmation ions due to deposits in the ion source or other instrument maintenance, it was not practically feasible to create a fixed confirmation ratio between batches. Thus, the confirmation ratio had to be adjusted, as indicated previously, based on the calibrators from each analytical batch.

Validation of this analytical method of solid-phase extraction, reversed phase HPLC, and isotope dilution tandem mass spectrometry included 1. determination of the method limit of detection, 2. characterization of the accuracy and reproducibility of the results from the analysis of quality control materials, 3. measurement of the level of the analytes of interest in urine after exposure of an animal model, and 4. application of the method to people who with no known exposure to the toxins abrin and ricin.

The calibrators from 14 separate analytical batches were used to determine the method limit of detection for each analyte, 0.09 and 0.08 ng/mL for L-abrine and ricinine, respectively. No more than two analytical batches were prepared on any single day in order to incorporate the normal daily variance that can result from instrument maintenance, temperature or slight changes in preparation routines. These limits of detection are sufficient to make this method useful for quantifying both expected background levels of L-abrine, as well as concentrations associated with an exposure (see Animal exposure study and background levels of L-abrine and ricinine in the general population).

Although there have been no human abrin exposures which have been evaluated with this biomarker method, the method is promising in light of a recent castor bean ingestion and the application of ricinine as a biomarker for ricin exposure (32). In this case, a 75-kg male consumed 6 castor seeds prior to seeking medical treatment with non-life-threatening symptoms. Urinary ricinine concentrations were quantified for up to 63 h post-exposure, at decreasing concentrations until an end-point of 130 ng/mL. This end point was more than 1000 time greater than the method limit of detection, and represented a non-life-threatening level of exposure. Thus, in a poisoning event, the analytical data generated by this method can be used to identify those who are exposed and those who are not exposed, focusing limited healthcare resources on those who need them.

Establishing quality control limits is a critical part of any routine assay to establish the acceptability of each batch which contains unknown samples. Quality control materials included a low- and high-enriched urine pool as well as a bench check. The bench check solution was a matrix plus internal standard spike, commonly referred to as a blank. The intensity of the internal standard in the bench QC, as well as peak retention time, served to indicate the batch recovery and instrument performance.

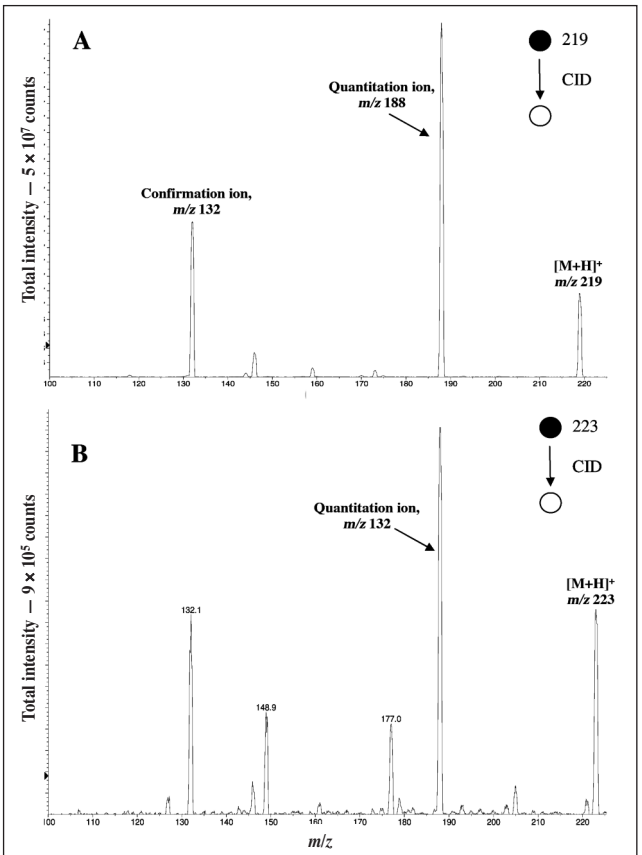


Figure 2. Electrospray production ion mass spectra native (A) and $^{13}C_1{}^2H_3$-labeled L-abrine.

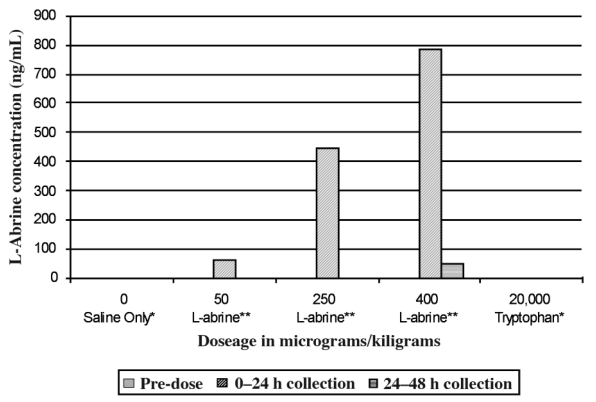
Table I. Characterization of Quality Control (Urine) Pools

Analyte	Pool	Concentration (ng/mL)	Mean (ng/mL)	Standard Deviation (ng/mL)	Coefficient of Variation (%)	Accuracy of Mean (%)
L-Abrine	Low	9.0	9.0	0.41	4.6	100
	High	160	162	12	7.5	101
Ricinine	Low	0.50	0.53	0.075	14	106
	High	50	51	0.40	0.78	102

The accuracy of the method, as determined by analysis of the enriched QC samples (see Table I), ranges from 100 to 106% and shows that the method is capable of accurately determining concentrations of abrine and ricinine in urine at the levels of most concern. The coefficient of variation (CV) is 7.5% or less except for the ricinine measurement at a spike level of 0.5 ng/mL, for which it is 14%. The higher CV is likely due to the low level of this spike which is only 6 times the detection limit. For cases of human exposure in which the urine concentration will depend on the relative ratio of biomarker and toxin, the route and the time after exposure, and the metabolism of the individual, the accuracy and precision of this method are adequate to provide an estimate of internal dose following exposure to these toxins.

Animal exposure study

An animal study was designed to monitor the excretion and recovery of L-abrine in 20 rats. In the absence of human clinical samples and relevant studies in the literature, this animal exposure study was carried out to provide insight that would not otherwise be available. The animals were exposed to one of three concentrations of L-abrine, a single high concentration of L-tryptophan, or no agent (control). The low L-abrine dose corresponded to 0.63*$LD_{50\ i.p.}$ abrin in mice (12), assuming a concentration ratio of 1:4 of abrin to L-abrine and an LD_{50} for abrin of 20 µg/kg. The mid and high L-abrine (250 and 400 µg/kg) doses were included to ensure that the target analyte could be detected and tracked over the course of the experiment. Figure 3 shows that L-abrine was predominantly excreted in the first 24 h after exposure. Quantification was possible for the first 24 h at all three dose levels. However, at 48 h, L-abrine in urine samples from rats exposed to the low and mid-level doses were below the method LOD. The rate of biomarker excretion is very important to know when collecting specimens, and as these results indicate, the most reliable and sensitive time period to collect urine is within the first 24 h post-exposure.



*There were no quantifiable levels of L-abrine in the control animals (saline dosed), or the tryptophan dosed animals.

**L-Abrine was not quantifiable beyond 24 hours for any dosage, except 400 micrograms per kilogram.

Figure 3. Analysis of rat urine after L-abrine dose.

Another issue associated with the use of L-abrine is its similar structure to tryptophan. In order to determine if tryptophan was a possible interference to L-abrine, a single large dose of tryptophan was administered to four rats at a level 50 times higher than the highest L-abrine dose. No L-abrine was detected in the urine from this group of animals indicating that tryptophan is not metabolized to L-abrine in rats and does not interfere with L-abrine determination using this method.

The amount of L-abrine recovered in the urine indicates the overall efficiency of the route of excretion and directly impacts the overall sensitivity of the assay for determining exposure. The total L-abrine recovery was calculated by comparing the mass of L-abrine injected in each rat and the mass recovered via urinary excretion. The mass in the urinary excretion was calculated from the product of the urine volume multiplied by the L-abrine concentration. The majority of the total L-abrine that entered the urine was excreted within 24 h, as previously reported (26). However, the amount of L-abrine recovered in this study was much lower than previously reported (26), between 2.72 and 4.07%. The 24–48 h urine collection data were not included in the recovery calculations because the levels had essentially returned to the background pre-dose levels of L-abrine after 24 h. It is possible that the lower total excretion is simply an artifact of the lower dose levels (500–1000 times less) used here as compared to previous experiments (26). It is also possible that the biomarker may have been metabolized to lower molecular weight products, the biomarker may be stored in tissue or that L-abrine was primarily excreted via another route from the body.

It should also be noted that the route of administration was a key factor in this animal study. Because the goal was to identify whether or not the metabolite can be quantified in urine, and a consistent route of introduction was required, intramuscular injections were used. Injections have different LD_{50} concentrations than via other routes and cannot be directly correlated to ingestion or inhalation.

These experiments did show that the biomarker L-abrine can be monitored in urine and is predominantly excreted in rats in the first 24 h after exposure. Because most of the biomarker is excreted rapidly, the study also indicates that sensitivity for this biomarker is highest in the first 24 h after exposure, and a low level abrin exposure up to 3 times the LD_{50} may not be detectable beyond 24 h post-exposure.

Table II. Statistical Comparison of Abrine and Ricinine in Urine and Water

		Slope		Intercept		
Analyte	Matrix	Estimate ± SE*	*p*-value	Estimate ± SE	*p*-value	R^2
Abrine	Urine	0.98 ± 0.003	< 0.0001	1.51 ± 0.35	< 0.0001	0.999
	Water	0.98 ± 0.002	< 0.0001	1.31 ± 0.33	< 0.0001	0.999
Ricinine	Urine	0.98 ± 0.004	< 0.0001	1.39 ± 0.51	0.0070	0.997
	Water	0.98 ± 0.003	< 0.0001	1.44 ± 0.40	0.0004	0.998

* SE = standard error.

Background levels of L-abrine and ricinine in the general population

Two main steps were required for quantifying L-abrine in the general population: 1. resolving the issue of background L-abrine levels in the urine pool used to validate the method and 2. analyzing the samples using that matrix. Because of the background level of L-abrine in urine from the general population, reproducibility and accuracy of the matrix-based calibration curve at sub-5 ng/mL concentrations were problematic. For quantification of low-level background concentrations in the urine of people with no known exposure, it was important to have an uncontaminated and consistent matrix into which standards could be spiked. As a result, we used deionized water as the standard matrix in order to more accurately quantify L-abrine.

In order to assess the accuracy while using water-based standards to quantify urine samples, a statistical comparison was made between 20 analytical calibration batches diluted in a urine matrix and 20 analytical calibration batches diluted in deionized water. Table II shows the results of weighted linear least-squares regression analysis of calibration curves for each matrix and analyte. The intercept and slope (which also describe method sensitivity) are of particular importance in comparing this analytical method in both urine and water. The intercepts for ricinine were the same in urine or water, but the intercepts for L-abrine were slightly offset because of the low level analyte contribution from the urine pool. The regression lines for urine and water for each analyte were compared to determine whether the slopes and regression lines were statistically different using methodology described by Neter and Washerman (33). For L-abrine, the difference between slopes was –0.0027 (95% CI: –0.0095–0.0041), and the regression lines were found to not be statistically different (p = 0.74). Thus, the regression lines determined in the two matrices were parallel, and for L-abrine, water and urine are equivalent as the calibration matrix. For ricinine, the difference between slopes was 0.0009 (95% CI: –0.0096–0.0114), and the regression lines were found to not be statistically different (p = 0.99). Thus, the regression lines for ricinine in water and urine were parallel and these two matrices can be used interchangeably for calibration.

The actual analysis of a reference range of human urine samples included 113 randomly collected specimens from people with no known exposure to abrin or ricin. Endogenous contributions to L-abrine were measured in 87% of the samples, at an average level of 0.72 ± 0.51 ng/mL (mean ± one standard deviation). The creatinine-corrected value of the reference range is 0.49 ± 0.44 µg L-abrine/g creatinine. The source of the background L-abrine level is not known, but may be due to interference or a metabolic product found in human urine. The animal exposure study, described in the previous section, does not support the production of L-abrine from tryptophan, but we currently are not aware of any other sources of L-abrine. Likewise, previous studies on the metabolism of L-abrine indicate that it has identical metabolic products to those from tryptophan, but do not identify other sources of L-abrine (31).

The results of this human reference range study shows that L-abrine can be detected in people with no known exposure to the toxin abrin. The accurate assessment of normal background levels of a biomarker is critical in interpreting analytical data in case of a suspected exposure. Because an upper 99% confidence limit of the unexposed population is 2.25 ng/mL (1.81 µg L-abrine/g creatinine), levels determined in urine at this value or lower cannot be used as evidence of an exposure to abrin.

Considering the results from both the human reference range and animal exposure studies, it is evident that L-abrine can be detected in urine at sub-lethal and acute exposure to abrin in the first 24 h. During the first 24 h after exposure, urinary concentrations resulting from exposure to a level which would be 63% of the toxin abrin LD_{50} would be expected to be at least 20 times the 99% confidence limit of the reference range. Exposure to abrin at levels which are equivalent to 5 times its LD_{50} would result in urinary concentrations of L-abrine greater than 400 times the reference range levels. However, beyond 24 h, the background levels in the general population may prevent further monitoring because they may no longer be higher than the range found in the unexposed population.

Ricinine was also monitored in this reference range study, and two positive ricinine results were detected where none had been previously reported (29). It is likely that these two positive results, which were below 4 ng/mL, indicate that the person was exposed to a product made from the Castor bean plant such as any one of numerous castor oil products (34). These two positive ricinine results equate to a detectable level of about 2% of the samples measured. Further studies will be carried out to better characterize this reference range for ricinine.

Conclusions

A new method is described here which uses biomarkers to quantify exposure to abrin or ricin. The method relies on the presence of an impurity from the original plant seed which is expected to be present in whole seeds or crude preparations. The biomarker L-abrine is excreted intact, primarily in the first 24 h post-exposure. Potential applications of this method include analyzing urine samples from people who may have accidentally or intentionally ingested rosary peas or castor seeds or their crude sample preparations. It is also applicable to incidents in which rosary peas or castor seeds are used to produce materials for poisoning. But, because a component of the source material instead of the intact toxin is quantified, exposure from a toxin not containing these impurities cannot be quantified using this approach.

Using urinary biomarkers in lieu of active toxins for identification of people exposed to the toxins has two main advantages. Because the intact toxins are primarily metabolized into lower molecular weight substances before they are transported into urine (17), the preferred matrix for measurement is blood. Urine collection is less invasive than blood collection and less objectionable to patients. Urinary biomarkers also provide an advantage in possible toxin exposures where the toxin route may not be known or cannot otherwise be collected without

difficulty, as would be the case in the eyes, intestinal tract, or lungs. Safety and regulatory issues are also significantly reduced when the handling of active toxins is not needed. Experimental procedures involving the biomarkers L-abrine and ricinine do not require select agent certification or special handling beyond the universal precautions needed when handling human urine.

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