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## Analysis of a Ricin Biomarker, Ricinine, in 989 Individual Human Urine Samples

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### Abstract

Ricinine (3-cyano-4-methoxy-N-methyl-2-pyridone) is a urinary biomarker which can be measured to confirm human exposure to castor bean products such as ricin. As many consumer products contain castor oil, another castor bean product, ricinine may be detectable in the general population. The following study characterized urinary ricinine concentrations from 989 individuals who were presumed to be unexposed to ricin. An automated diagnostic method was utilized here to simplify the analysis of this large sample set. Sample preparation included a 96-well polystyrene divinylbenzene high throughput extraction and preconcentration step. Purified samples were analyzed by an efficient dual column, reversed phase liquid chromatography separation and <sup>13</sup>C-isotope dilution tandem mass spectrometry. In this convenience sample, only 1.2% of the urine samples had detectable amounts of ricinine randomly distributed between 0.186 and 4.15 ng/mL.

### Keywords

Biological Markers; Urine; Chromatography; High Performance Liquid; Ricin; Poisoning; Diagnosis; Spectrometry; Mass; Electrospray Ionization

### Introduction

The castor bean plant, *Ricinus communis*, is the only known source of the toxin ricin (1). It is also the source of castor oil which is harvested to produce many beneficial products such as lubricants, pharmaceuticals, cosmetics, paints, and plastics (2; 3). Although an extremely potent protein toxin that is lethal to humans (4; 5; 6) and animals (7; 8; 9), ricin has also been investigated for use in the treatment of cancer (10; 11; 12) and AIDS (13; 10; 11). Active ricin is not present in castor oil as it is insoluble in oil (1), and the toxin is

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deactivated if the extraction is carried out under heated conditions such as those found in castor oil production (14). The small alkaloid ricinine (165 Da) is another component present in the castor bean and has been found in both ricin preparations and in castor oil (15). Detection of urine ricinine has been used as a biomarker of castor bean product exposure and has been used to confirm castor bean ingestion in suicide attempts (16).

Despite the many legitimate uses of the castor bean, ricin is an attractive toxin for terrorists (17). This is primarily due to the wide availability of the castor bean plant, the ease of toxin extraction from the seeds, and the high toxicity of the protein toxin. Castor seeds are harvested in Asia, Central and South America, Africa, and Europe with global castor bean production exceeding 1 million metric tons per year (<http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#anchor> accessed 03/02/2012; <http://www.crnindia.com/commodity/castor.html> accessed 03/02/2012) (15). Since ricin accounts for approximately 1–5% of the weight of the seed, there is the potential to produce 10,000 metric tons of ricin as a manufacturing by-product.

Inhalation and injection are the most potent means of ricin poisoning with estimated lethal doses ranging from 0.01 mg/kg to 1 mg/kg (15). The most common cause of ricin poisoning is ingestion of castor seeds despite being the least potent route of exposure (14). Intact castor seeds can pass through the digestive tract with minimal symptoms, and the level of seed mastication and maceration has a strong correlation with the observed toxicity of the ingested seeds (18, 16).

Ricin is an approximately 65 kDa glycoprotein (19; 20), and detection of the intact toxin using liquid chromatography tandem mass spectrometry (LC-MS/MS) without first performing a protein digest is not practical (21; 22; 23; 24). The small molecule ricinine (3-cyano-4-methoxy-N-methyl-2-pyridone) (Figure 1) has proven useful as a urinary biomarker for castor bean product exposure since it can be found in ricin preparations and is readily excreted after intoxication by ricin extracts. Ricinine (LD<sub>50</sub> 340 mg/kg i.p. in mice and 3 g/kg i.g. in mice) (25) can be measured non-invasively in urine via automated solid phase extraction (SPE) followed by LC-MS/MS analysis (15). Ricinine is unique to *Ricinus communis*, so its presence implies an exposure to a product derived from castor seeds which may include extracted ricin.

When the ricinine assay was first developed, only 30 individual specimens were analyzed in which no ricinine was detected (15). However, it was expected that ricinine concentrations should be detectable in some people since it was known that ricinine is present in consumer products that utilize castor oil. A follow-on expanded analysis was needed, and a group of 989 samples was selected for testing. The number of samples selected was based on a combination of factors including the laboratory's expanded capability to process samples quickly in a high throughput format and the fact that a large set of samples were readily available in house for testing. The results of the analysis of these urine samples and a brief description of the diagnostic method are presented in this paper.

## Experimental

### Chemicals and Reagents

Native ricinine standards in urine (calibrators: 0.300, 0.500, 10.0, 30.0, 100, 150 ng/mL; QC standards: 1.00 and 50.0 ng/mL) were purchased from Cerilliant Corporation (Round Rock, TX) and stored at  $-70^{\circ}\text{C}$  until needed. An additional calibrator, 0.0800 ng/mL, was prepared by dilution of the 0.500 ng/mL calibrator using DI water to use for bracketing unknowns with measured values below the normal 0.300 ng/mL calibration standard. Isotopically labeled ricinine ( $^{13}\text{C}_6$ ) in water (11.0 ng/mL) and blank urine was also purchased from Cerilliant and stored at  $-70^{\circ}\text{C}$ . These solutions are not available to the general public, but can be reproduced by following previously published methodologies (15). Human urine samples were purchased from Tennessee Blood Services (Memphis, TN). This study used urine acquired from commercial sources and thus the work did not meet the definition of human subjects as specified in 45 CFR 46.102 (f). Each sample was stored at  $-70^{\circ}\text{C}$ . HPLC-grade methanol, acetonitrile, and water were purchased from Tedia (Fairfield, OH). Fluka LC-MS grade formic acid was purchased from Sigma-Aldrich (St. Louis, MO).

### Sample preparation

All reference and unknown urine samples were aliquoted into 2 mL Nunc polypropylene round bottom 96-well microplates from Thermo Fisher Scientific (Rochester, NY). Internal standard was added manually following aliquoting into the 96-well plate. Manual addition of internal standard was needed to reduce waste when plates were not completely occupied with reference or unknown urine samples. Solutions were manipulated using 20–300  $\mu\text{L}$  single- and/or multi-channel micropipettes from Rainin (Oakland, CA), the Formatter Sample Processing Workstation from Tomtec (Hamden, CT), and the Sciclone i1000 Liquid Handling Workstation from Caliper Life Sciences (Hopkinton, MA). Solutions were mixed prior to aliquoting with a laboratory vortexer from Fisher Scientific (Pittsburgh, PA).

Each 96-well plate contained 200  $\mu\text{L}$  aliquots of the following: four spiked urine samples randomly added to the plate to ensure that LC-MS/MS results matched the predicted plate pattern; aliquots of each calibrator, blank and quality control standard; and up to 74 random unknown urine samples. This plating process was repeated for all 989 unknown urine samples. Each 96-well plate of unknowns, calibrators, and QC standards was considered an analytical batch.

Thirty microliters of  $^{13}\text{C}_6$ -isotopically enriched internal standard solution was added to each well, and the plate was mixed using a Labsystems Wellmix 96-well microplate mixer from Thermo Scientific (Waltham, MA). The plate was then transferred to the Caliper i1000 for vacuum extraction using a Strata-X, 33  $\mu\text{m}$  polymeric reversed phase 60 mg/well 96-well SPE microplate from Phenomenex (Torrence, CA). The sorbent bed was conditioned with 1150  $\mu\text{L}$  of methanol followed by 1150  $\mu\text{L}$  of HPLC grade water. Then, 150  $\mu\text{L}$  of each reference or unknown urine sample were loaded onto the sorbent bed. The sorbent bed was then washed using 1150  $\mu\text{L}$  of 5% methanol in water. Finally, 800  $\mu\text{L}$  of acetonitrile was used to elute the retained portion of the samples into 2 mL Nunc microplates.

The extracted samples were dried in the collection plates under nitrogen at 60°C using a Biotage (Charlotte, NC) TurboVap 96 Concentration Evaporator Workstation. Dried samples were reconstituted by adding 100 µL of HPLC grade water with the Caliper i1000 and vortexing the plate for one minute. Extracts were then transferred to an Advion PCR microplate, and the plate was sealed with a Thermo Scientific (Waltham, MA) Easy Pierce 20 µm foil heat seal (PN AB-1720).

### Instrumental Analysis

An Agilent Technologies (Santa Clara, CA) 1200 HPLC (two G1312A binary pumps, two G1379B degassers, one G1367B well-plate autosampler, and one G1316A column heating compartment equipped with an internal 10-port switching valve) was used for chromatographic separations. Twenty microliters of extract was injected onto 2×100-mm Polar RP Phenyl columns (Synergi, 2.5 µm, 100Å) from Phenomenex (Torrence, CA) with a 300 µL/min flow rate and 40°C column temperature. Two columns were utilized in parallel with one acting as the analytical column for the mass spectrometer while the other was being re-equilibrated. The two mobile phases were (A) 10% methanol in water with 0.019% formic acid, and (B) acetonitrile with 0.019% formic acid. The starting mobile phase concentration was 7% B. After 0.5 min, a gradient ramp to 50% B was initiated, ending at 2.0 min. These conditions were then held for one minute before returning to the original starting conditions for three minutes of re-equilibration (total LC run time is six minutes). At this point the 10-port valve was used to switch to the other column to initiate the next run.

An AB Sciex (Foster City, CA) API 4000 QTRAP triple-quadrupole mass spectrometer equipped with a turbo-ion-spray source was interfaced to the HPLC and operated in positive-ion multiple-reaction-monitoring (MRM) mode. The precursor/product ion transitions that were monitored were m/z 165/138 (quantitation), m/z 165/82 (confirmation), and m/z 171/144 (internal standard). Data analysis was performed using Analyst 1.4.2 instrument control software (AB Sciex, Foster City, CA). A 1/x-weighted linear least squares regression was applied to the standard concentration versus the area ratio of the quantification ion to the internal standard ion. A correlation coefficient ( $r^2$ ) of 0.990 or greater was used as a criterion for quantitation. A typical chromatogram for the lowest calibration standard is presented in Figure 2. The area ratio of the quantitation ion to the confirmation ion (i.e. confirmation ratio) was used as a criterion for specificity. The confirmation ratios for the calibrators in each analytical batch were averaged and a tolerance of +/-30% was applied to confirm all positive samples. An analytical batch consisted of 1 blank, 6 calibrators, 2 quality control (QC) materials, and any unknowns included on the microplate.

### Results and Discussion

The purpose of this study was to measure urinary ricinine concentrations from a large convenience sample group. To accomplish this, 989 individual specimens were extracted and analyzed for ricinine using an established method from this laboratory (15). The number of samples analyzed was based on the ability to process samples quickly, and that these samples were readily available for testing.

To ensure high quality results in this high throughput testing method, the following standard protocols were added to those of the manual method: 1) should one of the QC samples within a batch fail evaluation, the entire batch (96-well plate) including calibrators and unknown samples associated with that QC were re-aliquoted, re-extracted and rerun; 2) any sample with a positive result for ricinine was re-extracted and rerun to confirm the results; 3) if a sample did not have a minimum internal standard response of  $1.0 \times 10^4$  area counts, the sample was re-extracted and rerun.

To increase the throughput of analyzing this large set of unknown samples, two automated sample handling instruments were utilized in series. Each instrument was calibrated spectrophotometrically, with a NIST traceable reference, prior to use to ensure pipetting accuracy. Each instrument also contained at least one automated liquid sensing device to ensure each sample was processed correctly. Approximately 200 samples (three 96-well microplates) could be aliquoted in one day using the Tomtec Formatter. Those same samples could then be immediately extracted using the Caliper i1000 liquid handler and analyzed by the API 4000 Q-trap LC-MS/MS. This automation allowed the analyst to perform the solid phase extraction on one set of samples while simultaneously aliquoting another set.

Of the individual specimens analyzed, 1.2% contained detectable amounts of ricinine. Concentrations ranged from 0.186 to 4.15 ng/mL (see Table 1) with a method detection limit of 0.080 ng/mL. All samples that initially were positive for ricinine were rerun using an added calibrator at the method limit of detection. The lowest calibrator was selected to appropriately bracket two results which were observed below the lowest commercially produced calibrator. All positive results fell within the expected concentration range of ricinine 48 h after a lethal exposure to ricin (0.08 – 10 ng/mL) which assumes minimal ricin extraction (15). However, because ricinine is a separate alkaloid that is present in pharmaceutical and cosmetic products that contain castor oil constituents, and these results are within the expected range of a lethal exposure to ricin, the presence of ricinine as a marker for castor bean or ricin exposure needs to be interpreted in the context of other exposures in addition to whether it is consistent with the clinical presentation of a ricin exposure.

## Conclusion

Nine Hundred eighty-nine individual human urine specimens were analyzed by LC-MS/MS, and 12 of those samples (1.2%) were found to contain detectable amounts of ricinine. Health care professionals charged with evaluating a positive result for this assay should take into consideration the context of any positive ricinine results such as patient symptoms, patient exposure to unknown white powders, and patient use of castor oil or castor oil derived consumer products. Also, because analysis of these individual specimens found positive results not found in a smaller sample set, larger background analyses should be considered for any compound that, like ricinine, could reasonably be expected to be found in urine samples of the general population. A well designed automation process made such an expanded analysis a reasonable undertaking.

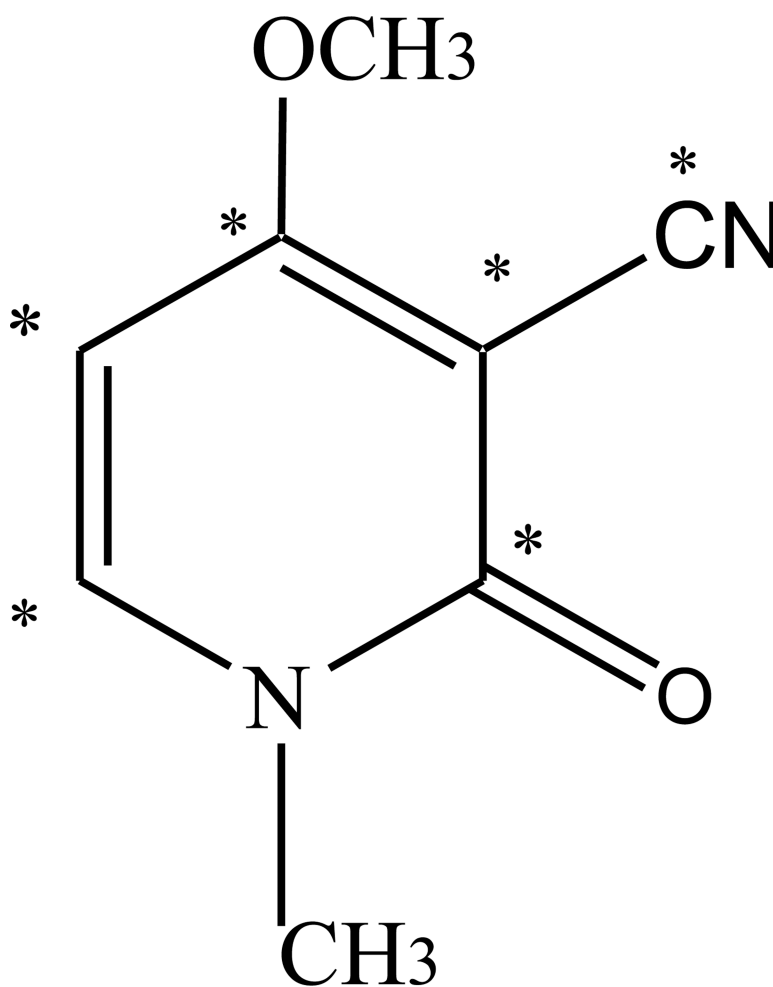
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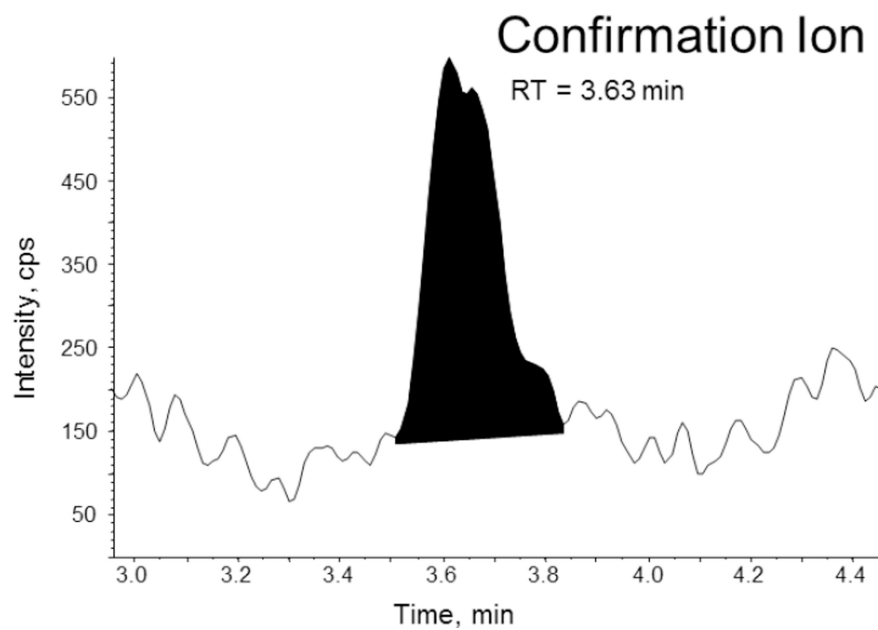
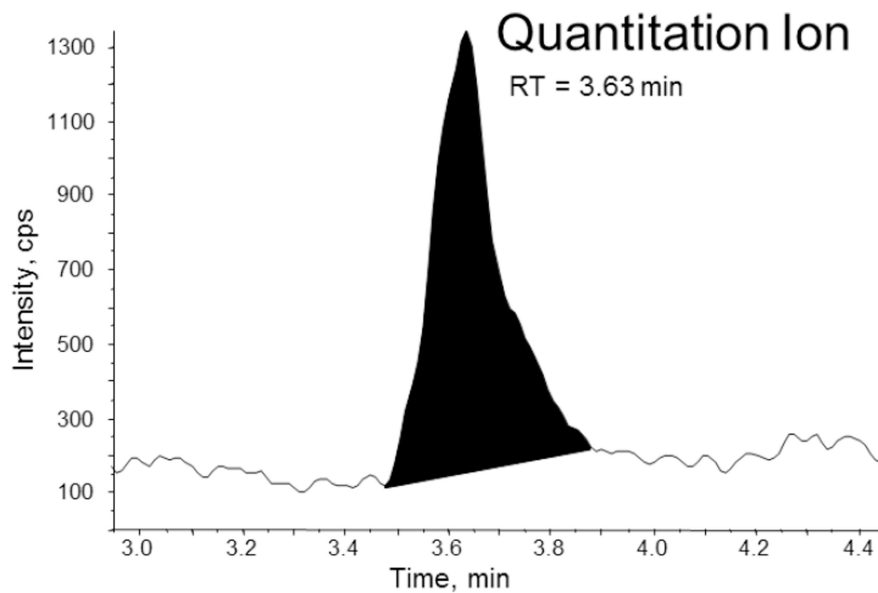
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**Figure 1.**  
Structure of Ricinine - Internal standard <sup>13</sup>C-labeling indicated by an asterisk





**Figure 2.** Typical Chromatogram of Ricinine: quantitation and confirmation transitions for 0.3 ng/mL standard ( $t_0 = 0.68$  min,  $k' = 4.33$ )

**Table 1**

Ricinine concentrations in samples with positive results

Sample ID	Ricinine (ng/mL)
1	4.15
2	3.19
3	0.325
4	0.278
5	3.35
6	0.487
7	0.438
8	0.423
9	0.186
10	0.491
11	0.863
12	0.466