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Toxicity and Detection of Ricin and Abrin in Beverages

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ABSTRACT

The oral and intraperitoneal (i.p.) toxicities to female BALB/c mice of ricin and abrin in phosphate-buffered saline (PBS), spring water, apple juice, and half-and-half (only oral) were examined after brief (2 h) and prolonged (11 to 13 days) storage. The ricin and abrin samples prepared in PBS had oral toxicities consistent with those previous studies, indicating oral and i.p. 50% lethal doses of >1 mg/kg of body weight and between 2 and 20 μg/kg of body weight, respectively. The toxicities of ricin and abrin in PBS were greater than those in apple juice and water. The oral toxicity of ricin and abrin in half-and-half appeared comparable to or less than that observed for the toxins in water. Spiked samples stored for a maximum of 11 days (13 for the abrin samples) at 4°C induced similar numbers of fatalities as did samples stored for only 2 h. Enzyme-linked immunosorbent assays of the samples administered by i.p. injection indicated a decrease in detectable toxin at 0.5 μg/ml.

Ricin and abrin are two members of class II ribosome-inactivating proteins derived from castor beans (*Ricinus communis)* and rosary peas (*Abrus precatorius*), respectively *(5, 8, 30–32).* Both toxins are composed of two subunits linked by disulfide bonds, an A chain and a B chain *(33, 34, 38).* The molecular masses of the two holo-toxins are comparable at approximately 64 kDa *(10).* The A-chains are *N*-glycosidases that catalyze the deadenylation of the 28S rRNA and thereby inactivate ribosomes and block protein synthesis *(14, 28, 31, 34, 35).* The B chains are lectins that direct the toxins to the cells and facilitate uptake *(19, 28, 31, 35).* Both toxins have been extensively characterized, and their x-ray structures have been determined *(4, 6, 9, 22, 29, 36).* Although abrin is homologous to ricin, antibodies raised against abrin do not cross-react with those against ricin, and vice versa *(11, 16).*

Depending on the cultivar, immunologically indistinguishable variants (isozymes) of ricin and abrin have been detected. In one purification procedure *(15),* abrin can be separated into three groups (I, II, and III). The three groups have different mouse intraperitoneal (i.p.) LD_{50} (50% lethal dose) values of 22, 2.4, and 10 μg/kg body weight *(15)* and an overall i.p. toxicity of 20 μg/kg body weight *(23).* The oral LD_{50} of ricin in rats and mice is 20 to 30 mg/kg body weight; the oral form is 1,000- to 10,000-fold less toxic than the injected (i.p. and intravenous) and inhaled forms *(2, 10, 20, 21).*

Castor beans and rosary peas also contain agglutinins that are immunologically cross-reactive with antibodies generated against the toxins (ricin and abrin) derived from the same source *(3, 11, 15).* The agglutinins are composed of two A chains and two B chains, have an approximate molecular mass of 120 kDa, and occur as multiple isozymes. Although agglutinin have not been as extensively characterized as have ricin and abrin, their physical properties and structures have been studied *(1, 18, 26, 37).*

Despite several cases of ricin intoxication involving oral administration, very little is known about its stability in food. The oral and i.p. toxicities of ricin, abrin, castor bean extract, and rosary pea extract stored in phosphate buffered saline (PBS), apple juice, half-and-half (a mixture that is half milk and half cream), and spring water were examined in female BALB/c mice. The ability to detect ricin and abrin also was examined using enzyme-linked immunosorbent assay (ELISA) technology.

MATERIALS AND METHODS

Ricin was purchased from Vector Laboratories (Burlingame, Calif.) and was used either as received (5 mg/ml) or after concentration to 21.5 mg/ml with an Amicon Centricon YM-30s ultrafiltration device (Millipore Corp., Billerica, Mass.). Abrin (fraction II) was prepared under contract to the U.S. Food and Drug Administration (FDA) according to the method of Hedge et al. *(15)* and purchased from Toxin Technologies (Sarasota, Fla.).

Preparation of extracts. Extracts of ground beans were made using PBS (catalog no. P3813, Sigma-Aldrich Corp., St. Louis, Mo.) supplemented with 0.1% Tween 20. The extract was concentrated by dialysis against solid PEG-14,000 (Sigma-Aldrich) at 4°C. The concentration of protein in the extracts was determined using the Coomassie plus protein assay (Pierce Biotechnology, Inc., Rockford, Ill.) adapted for use with a 96-well microtiter plate and read at 650 nm. The protein concentrations of the castor bean and rosary pea extracts were 38 ± 8 mg/ml (n = 6 dilutions, each in triplicate) and 15 ± 1 mg/ml (n = 6 dilutions, each in triplicate), respectively, with the extraction solvent (PBS plus 0.1% Tween 20) generating a background below the limit of quantitation (<0.1 mg/ml).

Quantitation of toxin in extracts. The concentrations of ricin and abrin in castor bean and rosary pea extracts, respectively, were determined using ELISAs (Tetracore, Inc., Gaithersburg, Md.) according to the manufacturer's recommended protocols. The samples were prepared in a mixture of 105 mM sodium phosphate, 75 mM

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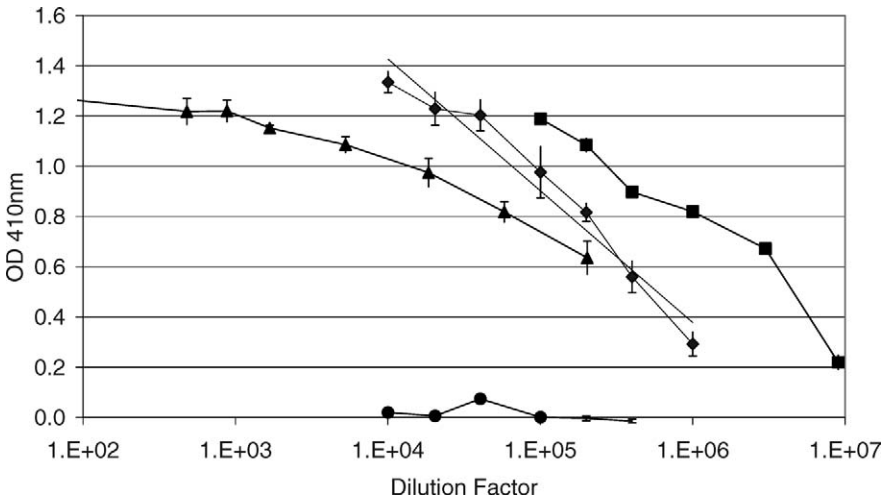

FIGURE 1. *ELISAs of ricin content of stock material used to spike beverages. Castor bean extract (▲), commercial ricin concentrated using the Centricon-30s system (■), filtrate from the centricon-30s system (●), and ricin standards (♦) were each serially diluted with UD (105 mM NaPi, 75 mM NaCl, 2.5% nonfat milk powder, and 0.05% Tween 20). A trend line described by* $y = -0.2279 \ln(x) + 3.5266$, *with* y *representing absorbance at 410 nm and* x *representing the concentration of ricin (nanograms per milliliter), agreed with the ricin standards with an* R^2 *value of 0.9576. The responses generated by the castor bean extract, concentrated commercial ricin, and filtrate were indicative of 0.7 ± 0.8 mg/ml ricin plus agglutinin (*n = 7*), 21.5 mg/ml ricin, and 0.1 mg/ml ricin, respectively.*

NaCl, 2.5% nonfat milk powder, 0.05% Tween 20 (UD) to minimize artifacts that may be due to nonspecific binding by lectins and to eliminate any food matrix effects on the assay *(12).*

Animal studies. The oral and i.p. toxicities of ricin, abrin, castor bean extract, and rosary pea extract were determined according to protocols approved by the Institutional Animal Care and Use Committee. Dosage groups of three female BALB/c mice were given oral doses (by gavage) or i.p. injections of 100 μl of ricin, abrin, castor bean extract, or rosary pea extract that had been stored for various time periods in PBS, water, apple juice, or half-and-half. Samples were prepared by spiking aliquots of each beverage at 22°C. The contribution by volume of the toxin solution (carryover) was a maximum of 25% for the oral samples containing 5.38 mg/ml ricin (dose of 21.5 mg/kg of body weight) and 0.58 mg/ml abrin plus agglutinin of rosary pea extract (dose of 2.3 mg/kg of body weight). Subsequent serial dilutions to prepare the various oral and i.p. (50, 5, and 0.5 μg/ml) samples appropriately reduced the level of carryover and potential impact on the solvent properties of the beverages. Samples were incubated at 22°C for 2 h and then transferred directly to a −80°C freezer or held refrigerated at 4°C and then transferred to the −80°C freezer. The samples were thawed immediately prior to administration to the mice, and the mice were monitored for 2 weeks. Handling, care, and monitoring of the BALB/c mice were conducted by Biocon, Inc. (Rockville, Md.) under contract to the FDA according to protocols approved by the Institutional Animal Care and Use Committees.

Statistical analysis of lethality data. Lethality data were analyzed according to two procedures. Cochran-Mantel-Haenszel (CMH) analyses were performed for stratified subgroups of the number of fatalities and the explanatory variables (storage time, purity, and beverage) after controlling for remaining explanatory variables. An analysis of variance (row mean score) was used when the explanatory variables were not on an ordinal scale and the ordinal mean number of fatalities was the column variable. Alternatively, a pairwise chi-square (χ^2) analysis was performed on the effects of storage, the purity of the toxin, and whether the toxin was dissolved in PBS, apple juice, water, or half-and-half on the survival data for each toxin. The Bonferroni inequality was used to adjust the χ^2-derived *P* values to account for multiple tests of the same data *(13).* The CMH and χ^2 analyses gave similar results, and the SAS system (SAS Institute, Cary, N.C.) was used to obtain more accurate *P* values for the CMH analysis.

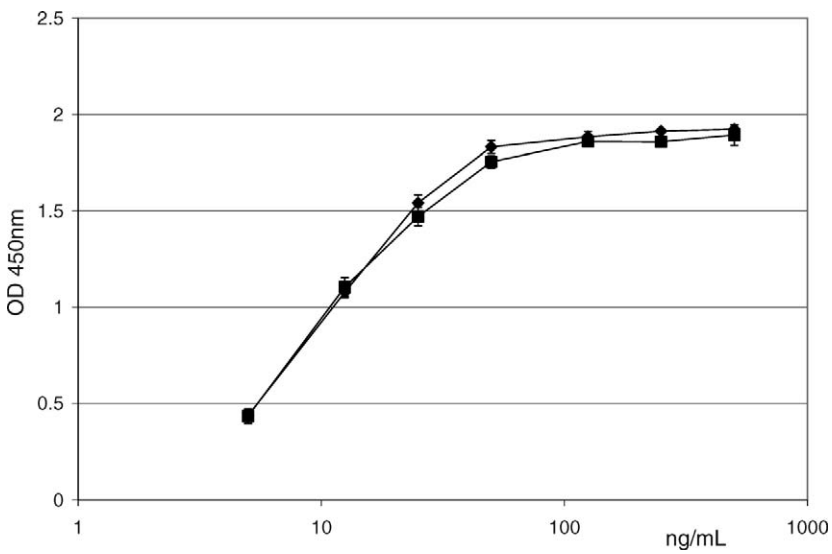

FIGURE 2. *ELISAs of ricin in PBS (♦) and UD (105 mM NaPi, 75 mM NaCl, 2.5% nonfat milk powder, and 0.05% Tween 20) (■). The error bars represent the standard deviations of the samples, which were processed in triplicate.*

TABLE 1. *Oral toxicity of ricin and abrin in female BALB/c mice*[a]

Beverage	Storage (h)[b]	Ricin Dose (mg/kg wt)	Ricin Mortality (%)[c]	Abrin Dose (mg/kg wt)	Abrin Mortality (%)	Beverage	Storage (h)[b]	Ricin Dose (mg/kg wt)	Ricin Mortality (%)[c]	Abrin Dose (mg/kg wt)	Abrin Mortality (%)
PBS	2	21.5	67	2.3[d]	33	Apple juice	2	21.5	33	2.3[d]	33
	46	21.5	67	2.3[d]	67		46	21.5	33	2.3[d]	0
	260 or 308	21.5	33	2.3[d]	33		260 or 308	21.5	100	2.3[d]	33
	2	7.2	33	1.05	33		2	7.2	33	1.05	0
	260 or 308	7.2	67	1.05	0		260 or 308	7.2	0	1.05	0
	2	2.4	33	0.77[d]	0		2	2.4	0	0.77[d]	0
	260 or 308	2.4	0	0.77[d]	33		260 or 308	2.4	0	0.77[d]	33
	2	0.3[e]	0	0.26[d]	0		2	0.3[e]	0	0.26[d]	0
	260 or 308	0.3[e]	0	0.26[d]	0		260 or 308	0.3[e]	0	0.26[d]	33
	2	0	0	0	0		2	0	0	0	0
	46	0	0	0	0		46	0	0	0	0
	260 or 308	0	0	0	0		260 or 308	0	0	0	0
Water	2	21.5	67	2.3[d]	0	Half-and-half	2	21.5	100	2.3[d]	67
	46	21.5	67	2.3[d]	0		46	21.5	67	2.3[d]	33
	260 or 308	21.5	100	2.3[d]	0		260 or 308	21.5	33	2.3[d]	33
	2	7.2	0	1.05	33		2	7.2	0	1.05	0
	260 or 308	7.2	0	1.05	0		260 or 308	7.2	0	1.05	0
	2	2.4	0	0.77[d]	33		2	2.4	0	0.77[d]	0
	260 or 308	2.4	0	0.77[d]	0		260 or 308	2.4	0	0.77[d]	0
	2	0.3[e]	0	0.26[d]	67		2	0.3[e]	0	0.26[d]	0
	260 or 308	0.3[e]	0	0.26[d]	0		260 or 308	0.3[e]	0	0.26[d]	0
	2	0	0	0	0		2	0	0	0	0
	46	0	0	0	0		46	0	0	0	0
	260 or 308	0	0	0	0		260 or 308	0	0	0	0

[a] Groups of three female BALB/c mice were given various doses of ricin and abrin. Each mouse received 100 ml of solution per 25 g of body weight by gavage.

[b] Ricin and abrin solutions were incubated for 2 h at 22°C and then stored at 4°C for 2, 46, or 260 (ricin and castor bean extract) or 308 (abrin and rosary pea extract) h and then frozen at −80°C. Solutions were thawed immediately before administration.

[c] Average mortality for groups of three female BALB/c mice over a 2-week period after administration of toxin.

[d] Detectable toxin (abrin plus agglutinin) level in rosary pea extract not corrected for agglutinin content.

[e] Detectable toxin (ricin plus agglutinin) level in castor bean extract not corrected for agglutinin content.

RESULTS AND DISCUSSION

Toxin content of castor bean and rosary pea extracts. Homology between the toxin and associated agglutinin made it impossible to distinguish and measure accurately toxin concentrations in extracts using available ELISA methods. Thus, only a composite estimate of toxin plus agglutinin (ricin plus agglutinin and abrin plus agglutinin) could be made. Analysis of castor bean and rosary pea extracts using commercial ELISAs with samples serially diluted over a 1,000-fold range indicated ricin plus agglutinin and abrin plus agglutinin concentrations of 0.7 ± 0.8 mg/ml ($n = 7$, each in triplicate) and 2.3 ± 0.5 mg/ml ($n = 4$), respectively. Ricin and abrin standards run alongside the samples were best described by trend lines of $y = -0.2279 \ln(x) + 3.5266$ ($R^2 = 0.9576$) and $y = 0.0429 \ln(x) - 0.0522$ ($R^2 = 0.994$), respectively, for toxin concentrations ranging from 5 to 500 ng/ml, with y representing absorbance at 410 nm and x representing the concentration (nanograms per milliliter) of the toxin. The titration curves for the samples of toxin in extract were not parallel to those for the purified toxins nor was it possible to fit the data to a single exponential processes, as illustrated in Figure 1 for the castor bean extract. In contrast, the slope of the line for ricin concentrated for use in the oral toxicity experiments was comparable to that of the line for the standard solution but appropriately shifted to higher dilution levels. The complex profile displayed by the extract, with a shallow curvature and slopes between adjacent data points that were less than the slope of the trend line describing the ricin standards, is characteristic of cross-reactivity of the ELISA with additional ligands with weaker binding affinities than those of ricin. The large variance in the calculated toxin content based on averaging serial dilutions is also characteristic of an ELISA cross-reaction with additional ligands such as agglutinin. The existence of multiple isozymes of the agglutinin and toxin make it problematic to extrapolate the curve obtained with extracts to values for dilutions for which binding of the toxin is the primary binding event. The average toxin plus agglutinin content calculated over multiple dilutions was used with its associated large standard deviation. The use of UD as the sample diluent for analyzing the extracts had no effect on the performance of the ELISA, which had a calculated limit of detection of 2 ng/ml (Fig. 2).

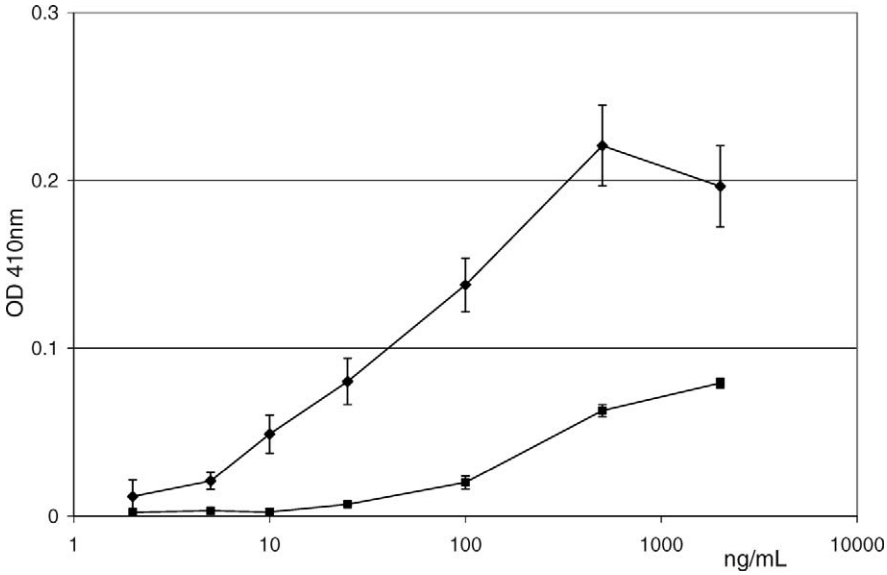

FIGURE 3. *ELISAs of abrin fraction II (♦) and abrin from Toxin Technologies in UD (105 mM NaPi, 75 mM NaCl, 2.5% nonfat milk powder, 0.05% Tween 20) (■). Error bars indicate the standard deviations for the samples analyzed in quadruplicate. The responses generated by 5 to 500 ng/ml abrin fraction II agreed with a trend line described by* y = 0.0429 ln(x) − 0.0522, *where* x *represents the concentration of abrin (nanograms per milliliter) and* y *is the absorbance at 410 nm, with an* R^2 *value of 0.994. Rosary pea extract diluted into UD displayed responses indicative of 2.3 ± 0.5 mg/ml abrin plus agglutinin (*n = 4*) (data not shown).*

TABLE 2. *Intraperitoneal toxicity of ricin, abrin, and castor bean and rosary pea extracts in female BALB/c mice*[a]

Beverage	Dose (μg/kg wt)	Storage (h)[b]	Mortality (%)					
			Ricin and castor bean extract			Abrin and rosary pea extract		
			Ricin	Bean extract[c]	Overall[d]	Abrin II	Pea extract[c]	Overall
PBS	200	2	100	67	84	100	100	100
	200	260 or 308	100	100	100	100	100	100
	20	2	100	100	100	100	100	100
	20	260 or 308	100	100	100	67	100	84
	2	2	67	0	34	0	33	17
	2	260 or 308	0	67	34	67	100	84
	0	2		0[e]	0		0	0
	0	260 or 308		0	0		0	0
Water	200	2	100	100	100	100	100	100
	200	260 or 308	100	100	100	100	100	100
	20	2	33	0	17	100	100	100
	20	260 or 308	100	100	100	100	100	100
	2	2	0	0	0	0	0	0
	2	260 or 308	0	0	0	0	0	0
	0	2		0	0		0	0
	0	260 or 308		0	0		0	0
Apple juice	200	2	100	100	100	100	100	100
	200	260 or 308	100	100	100	67	100	84
	20	2	100	100	100	33	100	67
	20	260 or 308	0	67	34	100	33	67
	2	2	33	0	17	67	0	34
	2	260 or 308	0	0	0	33	67	50
	0	2		0	0		0	0
	0	260 or 308		0	0		0	0

[a] Groups of three female BALB/c mice were given various doses of toxin or extract. Each mouse received 100 ml of solution per 25 g of body weight by i.p. injection and was monitored for 2 weeks.

[b] Ricin and abrin solutions were incubated for 2 h at 22°C and then stored at 4°C for 2 or 260 (ricin and castor bean extract) or 308 (abrin and rosary pea extract) h and then frozen at −80°C. Solutions were thawed immediately before administration.

[c] Extract administered based on detectable toxin content not corrected for agglutinin.

[d] Average mortality of groups administered comparable levels of detectable toxin as purified protein or extract.

[e] One group of three female BALB/c mice was used as a negative control for each beverage.

TABLE 3. *Toxicity in female BALB/c mice of ricin and abrin after prolonged storage*[a]

Toxin	Form	Dose (mg or μg/kg wt)[b]	Mortality: no. dead/no. given toxin stored for: 2 h	260 or 308 h	Overall[c]
Oral					
Ricin	Pure toxin	21.5	8/12	8/12	16/24
	Pure toxin	7.2	2/12	2/12	4/24
	Pure toxin	2.4	1/12	0/12	1/24
	Bean extract[d]	0.3	0/12	0/12	0/24
	Control	0	0/12	0/12	0/24
Abrin	Pea extract[e]	2.3	4/12	3/12	7/24
	Pure toxin	1.05	2/12	0/12	2/24
	Pea extract	0.77	1/12	2/12	3/24
	Pea extract	0.26	2/12	1/12	3/24
	Control	0	0/12	0/12	0/24
Intraperitoneal					
Ricin	Pure toxin	200	9/9	9/9	18/18
	Bean extract	200	8/9	9/9	17/18
	Pure toxin	20	7/9	6/9	13/18
	Bean extract	20	6/9	8/9	14/18
	Pure toxin	2	3/9	0/9	3/18
	Bean extract	2	0/9	2/9	2/18
	Control	0	0/9	0/9	0/18
	Pure toxin plus extract	200	17/18	18/18	35/36
	Pure toxin plus extract	20	13/18	14/18	27/36
	Pure toxin plus extract	2	3/18	2/18	5/36
Abrin	Pure toxin	200	9/9	8/9	17/18
	Pea extract	200	9/9	9/9	18/18
	Pure toxin	20	7/9	8/9	15/18
	Pea extract	20	9/9	7/9	16/18
	Pure toxin	2	2/9	3/9	5/18
	Pea extract	2	1/9	5/9	6/18
	Control	0	0/9	0/9	0/18
	Pure toxin plus extract	200	18/18	17/18	35/36
	Pure toxin plus extract	20	16/18	15/18	31/36
	Pure toxin plus extract	2	3/18	8/18	11/18

[a] Data for toxin dissolved in PBS, water, apple juice, and half-and-half were combined. Mice were monitored for 2 weeks after receiving toxin.
[b] Oral doses were milligrams per kilogram of body weight, and intraperitoneal doses were micrograms per kilogram of body weight.
[c] Total number of mortalities combining the results obtained with toxin stored for 2 and 260 (ricin) or 308 (abrin) h at 4°C.
[d] Ricin plus agglutinin.
[e] Abrin plus agglutinin.

Accurate determination of toxin concentration in the extracts was further limited by differences in the cross-reactivities of the isozymes *(7, 15, 17, 24, 27, 39),* as exemplified by the fivefold difference observed in the responses generated by abrin fraction II and commercially available abrin (Fig. 3). In previous research, abrin fraction II had an limit of detection of 2 to 5 ng/ml with the ELISA, which was comparable to that of abrin C (Sigma Chemical Co.) and lower than for abrin fraction I, abrin fraction III, and agglutinin *(11).* Thus, the use of abrin fraction II as a standard to calculate the content of rosary pea extract further served to provide an upper limit to the abrin content.

Toxicity. Tables 1 and 2 contain the itemized mortality results for the dosage groups of mice given ricin and abrin orally or by i.p. injection. The mortality data are consistent with published data indicating apparent oral and i.p. LD_{50} values for the toxins of approximately 5 to 20 mg/kg of body weight and 2 to 20 μg/kg of body weight, respectively. Although the low number of fatalities associated with oral administration of the toxins (doses that were lower than or equal to the oral LD_{50} values) might prevent detection of minor changes in toxicity, with the i.p. doses, which ranged from >10-fold to considerably less than the apparent LD_{50} values, it should be possible to detect changes in toxicity.

No significant difference was observed in the i.p. toxicity of either toxin regardless of whether it was administered as a pure protein or as an extract (CMH, $P = 0.7237$; χ^2, $P > 0.5$). Because oral toxicity studies require higher doses, differences observed between purified proteins and extracts may be due to more carryover.

Effects of storage on toxicity. The data presented in Tables 1 and 2 were recompiled based on duration of storage and beverage (Tables 3 and 4). No association was observed between duration of storage and the number of

TABLE 4. *Mortality in female BALB/c mice by toxin dose and beverage vehicle*[a]

Toxin	Dose (mg or μg/kg wt)[b]	Mortality (no. dead/no. given toxin)				
		PBS	Water	Apple juice	Half-and-half	Overall[c]
Oral						
Ricin	21.5	5/9	7/9	5/9	6/9	23/36
	7.2	3/6	0/6	1/6	0/6	4/24
	2.4	1/6	0/6	0/6	0/6	1/24
	0.3[d]	0/6	0/6	0/6	0/6	0/24
	0	0/9	0/9	0/9	0/9	0/36
Total fatalities		9	7	6	6	28
Abrin	2.3[d]	4/9	2/9	2/9	4/9	12/36
	1.05	1/6	0/6	0/6	0/6	1/24
	0.77[d]	1/6	1/6	1/6	0/6	3/24
	0.26[d]	0/6	1/6	1/6	0/6	2/24
	0	0/9	0/9	0/9	0/9	0/36
Total fatalities		6	4	4	4	18
Intraperitoneal						
Ricin[e]	200	11/12	12/12	12/12	ND[f]	35/36
	20	12/12	7/12	8/12	ND	27/36
	2	4/12	0/12	1/12	ND	5/36
	0	0/6	0/6	0/6	ND	0/18
Total fatalities		27	19	21	ND	67
Abrin[e]	200	12/12	12/12	11/12	ND	35/36
	20	11/12	12/12	8/12	ND	31/36
	2	6/12	0/12	5/12	ND	13/36
	0	0/6	0/6	0/6	ND	0/18
Total fatalities		29	24	24	NA[g]	77
Total fatalities		71	54	55		

[a] Data for toxin stored for various time periods were combined. Mice were monitored for 2 weeks after receiving toxin.
[b] Oral doses were milligrams per kilogram of body weight, and intraperitoneal doses were micrograms per kilogram of body weight.
[c] Overall mortalities combining the results for toxin administered in PBS, water, apple juice, and half-and-half at the specified dose.
[d] Ricin plus agglutinin in castor bean extract or abrin plus agglutinin in rosary pea extract.
[e] Composite of results from mice given toxin as a purified protein and as an extract (castor bean or rosary pea).
[f] ND, not determined.
[g] NA, not applicable.

mortalities (CMH, $P = 1$, χ^2, $P > 0.5$). Comparable mortalities or one-mouse differences were observed for ricin at various concentrations and different storage times. The largest change in abrin toxicity upon storage for 308 h (1 mouse versus 5 of 9 mice) was observed for rosary pea extract at 0.5 μg/ml abrin (dose of 2 μg/kg of body weight) administered by i.p. injection. The source data (Table 2) indicate that the increase in mortality for 0.5 μg/ml abrin (dose of 2 μg/kg of body weight) upon storage was due to an apparent increase in toxicity of the PBS samples. ELISA results for the PBS samples indicated comparable levels of detectable abrin after 2 and 308 h of storage. Thus, the change in mortality was not reflected in detectable abrin and is not currently understood. All but one of the remaining abrin samples displayed either comparable or singular differences in the number of fatalities, with the one remaining sample entailing a 2 of 12 change in fatalities.

Effects of PBS, water, apple juice, and half-and-half on toxicity. Because the duration of storage in the beverages did not affect toxicity, the data were pooled and compared based on dose and beverage (Table 4). The mortality observed at different doses for each beverage indicated more fatalities when the toxins were dissolved in PBS than when they were dissolved in water, apple juice, or half-and-half (half-and-half was administered only orally) (χ^2, $P < 0.1$). Results of the CMH analysis of the mortality associated with beverage were in agreement with those of the χ^2 test, with a P value of 0.0643. Subdivision of the data to compare the fatalities associated with water, apple juice, and half-and-half revealed no significant association between the three beverages and the number of fatalities due to oral administration of either ricin or abrin. When ricin was administered via i.p. injection, there was no significant association between the number of fatalities and whether the toxin was dissolved in water or apple juice (χ^2, $P > 0.25$). Compensatory differences were observed in the number of fatalities associated with i.p. administration of abrin at 20 and 2 μg/kg of body weight in water and apple juice, with no significant overall association between fatalities and whether abrin was dissolved in either beverage (χ^2, $P > 0.5$).

Although the differences between the number of fatal-

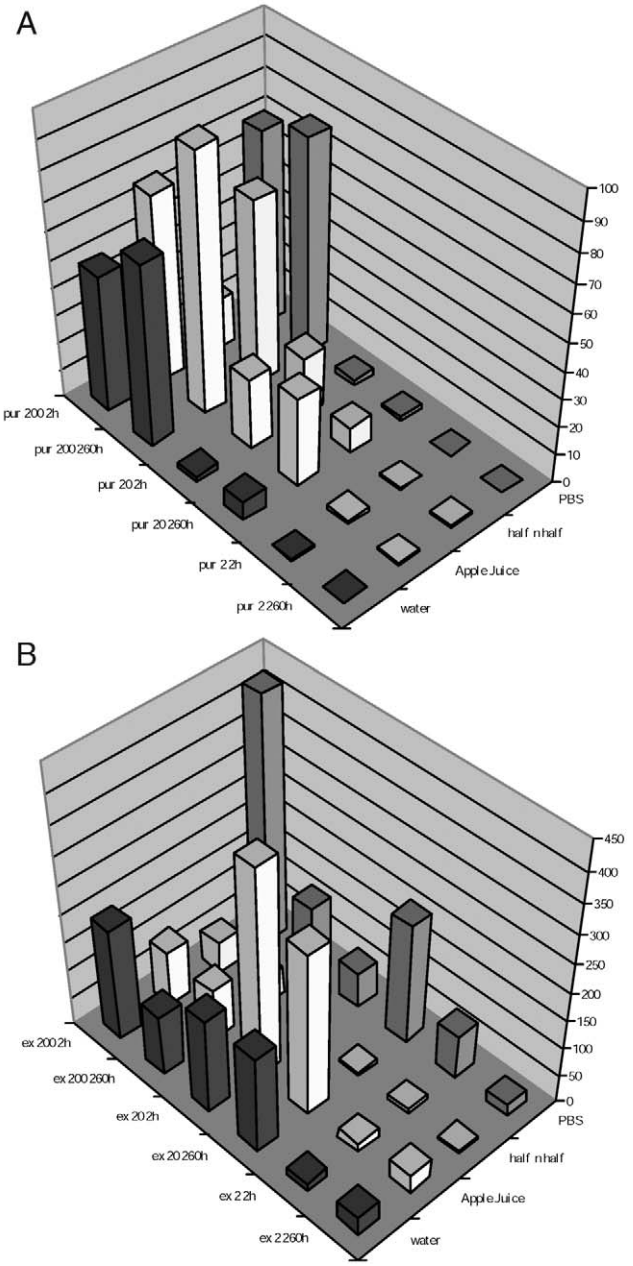

FIGURE 4. *ELISA detection of ricin or ricin plus agglutinin in (A) ricin and (B) castor bean extract i.p. samples. The ''200 pure 2h'' designation refers to i.p. samples made with purified ricin that was stored for 2 h and when administered at 100 μl/25 g of mouse body weight provided a ricin dose of 200 μg/kg of body weight. The ''2 ex 260h'' designation refers to i.p. samples made from castor extract that was stored for 260 h and when administered at 100 μl/25 g of mouse body weight provided a ricin plus agglutinin dose of 2 μg/kg of body weight. The i.p. samples were diluted to a final concentration of 50 ng/ml in 10 mM PBS plus 5% nonfat milk powder plus 0.1% Tween 20 immediately before analysis. Detectable ricin (and ricin plus agglutinin for castor bean extract) levels were based on ricin standards that were processed with the samples. The i.p. toxicity measurements were not conducted with the half-and-half samples, which were administered only orally.*

TABLE 5. *Number and distribution of fatalities in female BALB/c mice given 100-ml aliquots of oral or i.p. toxin*

Beverage	No. of fatalities[a]			No. of dosage groups with fatalities[b]		
	Oral	i.p.	Total	Oral	i.p.	Total
PBS	15	56	71	6	6	12
Water	11	43	54	4	4	8
Apple juice	10	45	55	5	6	11
Half-and-half	10	ND[c]	NA[d]	2	ND	NA

[a] Fatalities associated with each beverage irrespective of toxin or dose.
[b] See Table 4 for dosage groups.
[c] ND, not determined.
[d] NA, not applicable.

ities did not indicate a significant difference between water, apple juice, and half-and-half, similar trends were observed repeatedly between the three beverages. Abrin in apple juice administered orally at 2.3, 0.77, and 0.26 mg/kg of body weight and ricin in apple juice at 21.5 and 7.2 mg/kg of body weight resulted in fatalities. In contrast, ricin and abrin in half-and-half administered orally were toxic at only the highest concentrations administered, resulting in six and four of nine fatalities (66 and 44%), respectively. Ricin administered orally in water induced fatalities (seven of nine) at only the highest concentration. Oral administration of abrin in water had an effect similar to that of abrin administered in apple juice. The lack of a reduction in the oral toxicity of abrin in water may be due to use of rosary pea extract in three of the four samples, which because of carryover could stabilize the toxin from effects of the water. The i.p. data are consistent with this explanation. Extensive dilution of the i.p. samples should have eliminated any carryover. Neither ricin nor abrin at 2 μg/kg of body weight in water were toxic (0 of 12 fatalities), but fatalities were observed for ricin in apple juice.

Table 5 contains the number and distribution of fatalities for the different beverages and PBS. Because each beverage was spiked with toxin, handled, and administered to the mice in an identical fashion, the data were compared based on the total number of fatalities and total number of dosage groups that had fatalities. The toxins were significantly more toxic in PBS than in apple juice, water, or half-and-half (χ^2, $P < 0.1$). The distribution of fatalities by the number of dosage groups also indicated greater toxicity associated with dissolution in PBS versus apple juice, water, or half-and-half, with oral and i.p. administration each associated with fatalities in six different dosage groups. Toxin dissolved in apple juice resulted in fatalities in five oral dosage groups and in six groups given by i.p. injections. In contrast, toxin in water resulted in fatalities in only four dosage groups given toxin either orally or by i.p. injection. Toxin administered orally in half-and-half caused fatalities in only two dosage groups. Overall, five of the six classifications summarized in Table 5 agree with an order of toxicity of the spiked samples of PBS $>$ apple juice $\geq$ water $\geq$ half-and-half. The sixth classification would reverse the

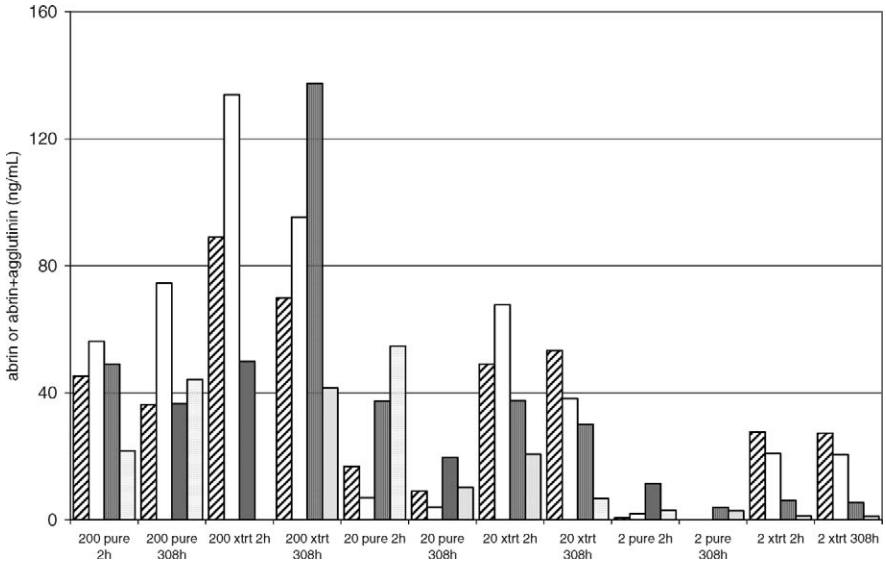

FIGURE 5. *ELISA detection of abrin or abrin plus agglutinin in i.p. samples. The ''200 pure 2h'' designation refers to i.p. samples made with purified abrin that was stored for 2 h and when administered at 100 μl/25 g of mouse body weight provided an abrin dose of 200 μg/kg of body weight. The ''2 xtrt 308h'' designation refers to i.p. samples made with rosary pea extract that was stored for 308 h and when administered at 100 μl/25 g of mouse body weight provided an abrin plus agglutinin dose of 2 μg/kg of body weight dose. The samples were prepared in PBS (▨), water (□), apple juice (■), and half-and-half (⬚). All samples were diluted to a final concentration of 50 ng/ml in 10 mM PBS plus 5% nonfat milk powder plus 0.1% Tween 20 immediately before analysis. Detectable abrin levels (and abrin plus agglutinin levels for rosary pea extract) were based upon abrin standards processed with the samples. The i.p. toxicity measurements were not conducted with the half-and-half samples, which were administered only orally.*

positions of apple juice and water but may reflect either the already discussed possible effect of carryover or overreliance on a single difference in the number of fatalities.

The mimicking of the overall trend by the beverages as indicated in Table 5 is consistent with the variables being independent and supports the prior assumption that the data could be collapsed to focus on the beverages by using a chi-square analysis.

Inability to distinguish between toxin and agglutinin. The inability of the ELISA to distinguish between toxin and agglutinin means that the toxin concentrations ascribed to the extracts were an overestimation. Thus, the observation that purified toxin and crude extracts administered via i.p. injection displayed similar toxicities is surprising. Possible explanations for the lack of difference in mortality rate between the purified toxin and the extract include (i) extract was more toxic and compensated for the reduction in toxin concentration, (ii) extract was predominately toxin, and (iii) differences between actual and calculated concentrations of toxin were insignificant relative to the 10-fold differences in concentration between the samples. The first explanation is consistent with the slightly higher mortality associated with oral administration of 0.19 mg/ml abrin as rosary pea extract (dose of 0.77 mg/kg of body weight) versus 0.26 mg/ml purified abrin fraction II (dose of 1.05 mg/kg of body weight). The recovery of twice as much toxin than agglutinin from castor beans *(25)* and rosary peas *(15)* is inconsistent with the second explanation but consistent with the hypothesis that the 10-fold decrease between doses had a more significant effect on toxin concentration than did the presence of agglutinin. Differences in growth conditions and between cultivars and selective pressures associated with the purification process may affect the isozyme distribution and recoveries, which in turn could create conditions favoring the first two explanations.

Toxin detection. Figures 4 and 5 depict the results of ELISAs conducted on the i.p. samples diluted to 50 ng/ml. As for the toxicity studies, no differences were observed upon prolonged storage. Although all of the samples were diluted to the same concentration of ricin (or abrin), the responses generated were not consistent. The pure toxin and extract samples had less detectable toxin with decreasing concentration of toxin in the original sample. The samples with the lowest concentration of pure toxin, 0.05 μg/ml (dose of 2 μg/kg of body weight) generated responses approaching the background. The high level of variance associated with the triplicate analyses (approximately 50%) made it impossible to determine whether the decrease in detectable toxin was associated with beverage dependence similar to the effects observed with toxicity (PBS > apple juice > half-and-half = spring water). It could not be determined whether the decrease in toxicity observed between different beverages represented changes in activity (Km) associated with the beverages or with a decrease in (detectable) toxin. The loss of detectable toxin at 0.05 μg/ml would be consistent with the loss of toxicity. Because the samples were analyzed in buffers known to stabilize the toxins, the decrease in detectable toxin was probably not readily reversible. It is not known whether the loss of toxicity associated with storage in different beverages was reversible.

The results of a commercial ELISAs of ricin and abrin in PBS, apple juice, half-and-half, and spring water provided a reliable indication of the amount of toxic ricin and abrin present each the beverage. Changes in toxicity were

reflected by the ability to detect the toxin. The stability of the toxin in the beverage did not significantly change over 11 days for ricin or 13 days for abrin during storage at 4°C. Differences were observed in the toxicity of ricin and abrin in PBS > apple juice ≥ water ≥ half-and-half.

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