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Assessing Risks of Plant-Based Pharmaceuticals: II. Non-Target Organism Exposure

Leslie M. Shama and Robert K. D. Peterson
Agricultural and Biological Risk Assessment, Department of Land Resources and Environmental Sciences, Montana State University, Bozeman, MT, USA

ABSTRACT

Plant-based pharmaceuticals potentially offer a cleaner method of producing a protein for drug manufacturing than traditional methods because plants are free of mammalian infectious agents. However, in the open environment they have the potential for intra-and inter-species gene flow, protein exposure to the public and non-target organisms, and they also have the potential to contaminate livestock feed. This study used probabilistic approaches to quantify the non-target organism risks associated with three pharmaceutical proteins produced in field-grown maize. The risk assessment for plant-based pharmaceuticals was conducted for four receptor species used as surrogates for a wider range of species. Body weights and maize consumption rates for each species were modeled from currently available information and used to calculate the exposure based on expression levels of three proteins. The acute dietary exposure for the receptor species was a single-day event in which the total maize consumption came from the recombinant maize. The non-target organism risk assessment demonstrated that risks will vary between species and between proteins, based primarily on differences in toxic endpoint and consumption rates. It also shows the utility of probabilistic, quantitative risk assessment methodologies and the importance of assessing risks from plant-based pharmaceuticals on a case-by-case basis.

Key Words: biotechnology, risk assessment, aprotinin, gastric lipase, *Escherichia coli* heat-labile enterotoxin B subunit (LT-B).

INTRODUCTION

Genetic engineering has made it possible to use plants as factories for pharmaceutical protein production. Plant-based pharmaceuticals are made by inserting a segment of DNA that encodes the protein of choice into the plant cells. The plants

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Address correspondence to Dr. Robert K. D. Peterson, Department of Land Resources and Environmental Sciences, Montana State University, 334 Leon Johnson Hall, Bozeman, MT 59717-3120, USA. E-mail: bpeterson@montana.edu

or the plant cells are essentially factories used to produce the desired proteins and are only grown for the purpose of pharmaceutical applications (Shama and Peterson 2004).

Plants expressing pharmaceutical proteins are currently being grown in field environments (although on few hectares and only in confined field trials) throughout the United States and other countries. Plant-based pharmaceuticals have the benefits of being less expensive to produce and potentially being more readily available to individuals in remote locations. It is also a cleaner method of producing a protein for drug manufacturing because plants are free of mammalian and avian infectious agents.

Maize is an attractive vehicle for orally administered, cloned vaccine antigens and other pharmaceutical proteins because it is capable of being processed into several palatable forms. Maize-based antigens are also inexpensive to produce and scale up. The distribution of the cloned antigen within the maize kernels is homogeneous, allowing for a reproducible dose (Fischer and Emans 2000). Such a vaccine would be well suited for developing nations where refrigeration during storage and distribution is often difficult, and syringes and other supplies for immunization are expensive and unsafe. The proof-of-concept for transgenic plant vaccines has been demonstrated in farm animal models and extensive trials are underway with promising results (Ma *et al.* 2003; Peterson and Arntzen 2004).

However, there are concerns about growing therapeutic proteins in the open environment. Plant-based pharmaceuticals in the environment have the potential for intra-and inter-species gene flow, protein exposure to the public and non-target organisms, contamination of livestock feed, and water contamination (Peterson and Arntzen 2004; Freese 2005).

Plant-based pharmaceutical production requires regulatory involvement of both the U.S. Department of Agriculture (USDA) and the U.S. Food and Drug Agency (USFDA), and this often involves risk assessment approaches. A risk assessment ensures a robust, transparent, and science-based process in which the assumptions and uncertainties associated with the assessment are considered and presented. Ecological risk assessments evaluate the likelihood that adverse ecological effects may occur or are occurring as a result of exposure to one or more stressors. Much attention has been directed toward the use of probabilistic risk assessment techniques that statistically quantify ecological risks as well as the associated uncertainty and variability in the subsequent risk conclusions (SETAC 1994; USEPA 1999). Risk assessment paradigms for genetically engineered plants do not differ in principle from those for other technological risks. Therefore, probabilistic approaches to quantify risks for crop biotechnology should be used for conducting ecological risk assessments, where appropriate (NRC 2000; Wolt and Peterson 2000; Wolt *et al.* 2003).

The objective of our study was to use probabilistic approaches to quantify nontarget organism dietary risks associated with three pharmaceutical proteins produced in field-grown maize. Risks from three proteins occurring in field-grown maize were evaluated based on a single exposure scenario, and the potential risks were compared between species and proteins.

MATERIALS AND METHODS

Problem Formulation

The non-target organism dietary risk assessment for plant-based pharmaceuticals was conducted for four receptor species used as surrogates for a wider range of species. The four receptor species chosen were meadow vole (*Microtus pennsylvanicus*), bobwhite quail (*Colinus virginianus*), whitetail deer (*Odocoileus virginianus*), and feeder and slaughter cattle (*Bos taurus)*. Body weights and maize consumption rates for each species were modeled from currently available information and used to calculate the exposure based on different expression levels of the proteins. The acute dietary exposure for the receptor species was a single-day event where the total maize consumption came from the recombinant maize. Body weights, consumption rates, protein expression levels, and total protein exposures were determined using quantitative, probabilistic methods.

Effects Assessment

More general information about the proteins, including their therapeutic activity, is presented in Shama and Peterson (*this issue*).

Aprotinin toxic endpoint

Acute LD_{50} values for mice, rats, dogs, and rabbits have been determined, but only for injections of aprotinin (Bayer Pharmaceuticals Corporation 2003). LD_{50} values were 910 mg kg^{-1} body weight (BW) for mice, 700 mg kg^{-1} BW for rats, 190 mg kg^{-1} BW for dogs, and 70 mg kg^{-1} BW for rabbits. The toxicity endpoint for the risk assessment was based on the no-observed-effect-level (NOEL) for dogs injected with aprotinin at 140 mg kg^{-1} BW (Trautschold *et al.* 1967).

Gastric lipase toxic endpoint

The ingestion no-observed-adverse-effect-level (NOAEL) for gastric lipase was 1000 mg kg^{-1} BW in rats (Coenen *et al.* 1997). After a 13-week oral toxicity study in rats by ANZFA (2002), an ingestion NOEL of 830 mg kg^{-1} BW was determined. Greenough *et al.* (1996) also support the 1000 mg kg^{-1} BW ingestion NOEL. The maximum NOAEL used in their 13-week oral toxicity study in rats was 1350 mg kg^{-1} BW. The toxicity endpoint for our risk assessment was 1000 mg kg^{-1} BW.

E. coli heat-labile enterotoxin B subunit (LT-B) toxic endpoint

When LT-B was administered to adult female mice (30 g BW) through intragastric administration, Guidry *et al.* (1997) observed a NOEL of 125 μg of LT-B. Therefore, a NOEL of 4.2 mg kg^{-1} BW was used for our assessment.

Exposure Assessment

More general information about each protein's expression in maize is presented in Shama and Peterson (*this issue*).

Protein Expression Assumptions

Based on known and predicted levels, aprotinin expression level was evaluated at 100 mg kg^{-1}, LT-B expression was at 500 mg kg^{-1}, and gastric lipase expression was at 1000 mg kg^{-1}.

Exposure Assumptions

Choice of surrogate species

The surrogate species were chosen to represent a relatively broad range of species (livestock and wildlife) that potentially could ingest the pharmaceutical protein expressed in maize kernels. The surrogate species included: feeder and slaughter cattle, whitetail deer, meadow vole, and bobwhite quail.

Routes, pathways, and durations of exposure

The exposure assumptions were that the entire daily food intake for each receptor species came from the transgenic maize kernels expressing the therapeutic protein and this was their sole source of food. The exposure duration was acute, occurring only over one day. Other exposure routes, such as inhalation of pollen and ingestion of leaves, stalks, and roots were not considered because it was assumed that each pharmaceutical protein would be produced only in the kernels. For cattle and whitetail deer, it was assumed that individuals entered the field and fed on the kernels, as they were mature or maturing while still on the ears. For voles and quail, it was assumed that individuals fed on kernels that may have dropped from the ears during or just before harvest.

Probabilistic exposure assessment

A Monte Carlo simulation model (Crystal Ball 2000 ver. 5.2, Decisioneering, Denver, CO) was used to determine protein exposures to surrogate species. The simulation was set to perform 5000 iterations for distributional analysis using several input assumptions (Table 1). Monte Carlo simulation uses random numbers to measure the effects of uncertainty and variability in a spreadsheet format. The simulation uses a probability distribution function from each input variable to randomly select values and repeatedly selects values based on their frequency of occurrence in the distribution. The variability for each input is taken into account in the output of the model so that the output is itself a distribution and reflects the probability of values that could occur.

The animal body weights and food consumption rates for each surrogate species were defined with a probability distribution based on the most relevant data (see later) (Table 1). To calculate the protein exposure for each surrogate species, the following equation was used:

$$IE = (PE * FC) \div BW \tag{1}$$

where IE = Ingestion Exposure (mg protein kg body $weight^{-1}$ day^{-1}), PE = Protein Expression (mg kg $kernel^{-1}$), FC = Food Consumption (kg dry weight of kernels day^{-1}), and BW = body weight (kg).

Table 1. Input distributions for the probabilistic exposure analysis.

Input distribution	Distribution type	Parameter	Value	Unit
			Aprotinin = 100 Gastric Lipase = 1,000	
Protein expression level	Normal	Mean	LT-B = 500	mg kg^{-1}
		SD	20	
		Lower Bound	0	
Feeder cattle				
Consumption	Normal	Mean	7.14	kg day^{-1}
		SD	0.36	kg day^{-1}
		Lower Bound	0	
Weight	Normal	Mean	316.64	kg
		SD	63.52	kg
		Lower Bound	0	
Slaughter cattle				
Consumption	Normal	Mean	7.14	kg day^{-1}
		SD	0.36	kg day^{-1}
		Lower Bound	0	
Weight	Normal	Mean	532.4	kg
		SD	46.93	kg
		Lower Bound	0	
Vole				
Consumption	Normal	Mean	6.1	g day^{-1}
		SD	0.8	g day^{-1}
		Lower Bound	0	
Weight	Normal	Mean	29.2	g
		SD	0.4	g
		Lower Bound	0	
Whitetail deer				
Consumption	Triangular	Minimum	2.02	kg day^{-1}
		Likeliest	2.27	kg day^{-1}
		Maximum	2.52	kg day^{-1}
Weight	Triangular	Minimum	40.9	kg
		Likeliest	88.64	kg
		Maximum	136.36	kg
Bobwhite quail				
Consumption	Normal	Mean	14.74	g day^{-1}
		SD	1.8	g day^{-1}
		Lower Bound	0	
Weight	Normal	Mean	191.26	g
		SD	9.3	g
		Lower Bound	0	

Protein expression

Protein expression was the amount of protein expressed in the maize kernels. Three mean expression levels, 100 mg kg^{-1}, 500 mg kg^{-1}, and 1000 mg kg^{-1}, were used for aprotinin, LT-B, and gastric lipase, respectively. Expression levels were assumed to be normally distributed with a standard deviation of 20% (Table 1).

Cattle weight and feed consumption rate

The cattle weights used for both feeder and slaughter cattle were derived from individual weight records separated by state (USDA 2006). The states chosen were located in the Midwestern United States and included Illinois, Nebraska, South Dakota, Missouri, Oklahoma, Kansas, and Iowa. There were no data for slaughter cattle for South Dakota, but all other states were included in the slaughter cattle calculation. A weighted mean and a standard deviation were calculated for both feeder and slaughter cattle.

The weighted mean for body weight was calculated by dividing the number of cattle measured in each state by the total number of cattle measured in all states, and then multiplying this value by the average weight from each state. The final mean was obtained by adding the values. The feeder cattle weight mean was 316.64 $\pm$ 63.52 kg and the slaughter cattle weight mean was 532.4 $\pm$ 46.93 kg. The weights for both were normally distributed (Table 1).

The feed consumption rate was determined for both feeder and slaughter cattle by calculating a weighted mean based on values from a maize feeding study (Loerch 1996). The same equation used to calculate body weight was used to calculate a weighted consumption mean. The feed consumption rate mean was 7.14 $\pm$ 0.36 kg for both feeder and slaughter cattle and the data were normally distributed (Table 1).

Vole weight and feed consumption rate

Vole weight was derived from Krol *et al.* (2004) by calculating a weighted mean as described earlier. The mean weight was 25.6 $\pm$ 3.7 g. Food consumption rate was also determined by calculating a weighted mean from the values presented in Krol *et al.* (2004). The weighted food consumption mean was 6.1 $\pm$ 0.8 g. Data for both food consumption rate and body weight were normally distributed (Table 1).

Bobwhite quail weight and feed consumption rate

The weight and food consumption rate of the bobwhite quail were obtained from the U.S. Environmental Protection Agency's (USEPA's) Wildlife Exposure Factors Handbook (USEPA 1993). The mean body weight was 191.26 $\pm$ 9.3 g and the food consumption rate mean was 14.74 $\pm$ 1.8 g. Data for both food consumption rate and body weight were normally distributed (Table 1).

Whitetail deer weight and feed consumption rate

Body weights for whitetail deer were calculated from a range of 40 to 136.36 kg. The range of distributions for body weight was obtained from Alabama Land Trust (2005) and Sedgwick County Zoo (2006). Weight was modeled as a triangular distribution with 88.64 kg as the likeliest weight (Table 1). Consumption rate was also modeled as a triangular distribution with 2.02 kg as the minimum rate, 2.27 kg as the likeliest rate, and 2.52 kg as the maximum rate (Table 1). These values were obtained based on the observed 2.27 kg day^{-1} food consumption rate (Gallagher and Prince 2003).

Risk Characterization

Risk quotients (RQs) were calculated for all three scenarios to integrate exposure and effect (toxicity). The dietary exposure values were determined probabilistically by Monte Carlo analysis. The exposure values were then divided by the toxic endpoint for each protein to determine the RQ. Therefore, the RQ, as used here, was the ratio between dietary exposure and the toxic endpoint.

RESULTS AND DISCUSSION

Aprotinin risk quotients (RQs), using the dog NOEL of 140 mg kg^{-1} BW for all of the ecological receptors and expression level of 100 mg kg^{-1}, ranged from 0.01 for slaughter cattle to 0.14 for voles at the 50th percentile of exposure. RQs ranged from 0.01 for slaughter cattle to 0.17 for voles at the 75th percentile. At the 90th percentile, RQs ranged from 0.01 for slaughter cattle to 0.2 for voles (Table 2). The vole had a higher RQ than the other surrogates because of the ratio between its body weight and consumption.

LT-B RQs, using a mouse NOEL of 4.2 mg kg^{-1} BW and expression level of 500 mg kg^{-1}, ranged from 1.59 for slaughter cattle to 24.85 for voles at the 50th percentile. At the 75th percentile, the RQs ranged from 1.72 for slaughter cattle to 27.12 for voles. At the 90th percentile, the RQs ranged from 1.84 for slaughter cattle to 29.24 for voles (Table 2).

Gastric lipase RQs, using a NOAEL of 1000 mg kg^{-1} BW for all of the ecological receptors and expression level of 1,000 mg kg^{-1}, ranged from 0.01 for slaughter cattle to 0.2 for voles at the 50th percentile. At the 75th percentile, the RQs ranged from 0.01 for slaughter cattle to 0.24 for voles. At the 90th percentile, the RQs ranged from 0.02 for slaughter cattle to 0.27 for voles (Table 2).

When comparing the RQs of all the proteins for all the ecological receptors, the RQs were highest for LT-B. The RQs under all the percentiles for LT-B were highest for vole and then quail. Gastric lipase had the lowest RQ values for all the ecological receptors. When examining gastric lipase alone, vole had the highest RQ values for

Table 2. Risk quotients from the probabilistic risk assessment.

Non-target organism	Percentiles								
	Aprotinin			Gastric Lipase			LT-B		
	50th	75th	90th	50th	75th	90th	50th	75th	90th
Quail	0.06[a]	0.06	0.07	0.04	0.04	0.05	18.55	20.24	21.69
Vole	0.14	0.17	0.20	0.20	0.24	0.27	24.85	27.12	29.24
Whitetail deer	0.02	0.02	0.03	0.03	0.03	0.04	3.04	3.62	4.35
Feeder cattle	0.02	0.02	0.02	0.02	0.03	0.03	2.69	3.11	3.67
Slaughter cattle	0.01	0.01	0.01	0.01	0.01	0.02	1.59	1.72	1.84

[a]Risk Quotient = Ingestion Exposure ÷ Toxic Endpoint.

Table 3. Non-target organism sensitivity analysis (percent contribution of variable to output variance).

Non-target organism	Consumption	Body weight	Protein expression
	Aprotinin Expression (100 mg kg^{-1})		
Vole	25.1[a]	4.4	70.6
Quail	28.5	0.3	71.3
Whitetail deer	2.5	55.8	41.7
Feeder cattle	2.9	49.3	47.8
Slaughter cattle	4.9	15.7	79.4
	LT-B Expression (500 mg kg^{-1})		
Vole	90.5	1.5	7.9
Quail	84.4	13.9	1.7
Whitetail deer	3.7	94.2	2
Feeder cattle	6.0	89.8	4.2
Slaughter cattle	20.4	66.9	12.7
	Gastric Lipase Expression (1,000 mg kg^{-1})		
Vole	97.5	1.0	1.6
Quail	81.7	11	7.3
Whitetail deer	2.3	96.8	0.9
Feeder cattle	6.2	92.7	1.1
Slaughter cattle	3.8	72.9	23.3

[a]Percent contribution of input variable to output variance.

all three percentiles (50th, 75th, 90th). The RQs for aprotinin were between the values for LT-B and gastric lipase, and, when examining the ecological receptors within aprotinin, vole had the highest RQ values.

The exposure distributions as a result of the Monte Carlo simulation for all of the receptor species, except whitetail deer, showed a normal distribution. The whitetail deer exposure distribution was lognormal with a flat kurtosis (4.37) and positive skewness (0.97). This most likely was the result of the triangular distribution and range of body weights used in the analysis. The exposure distribution for whitetail deer revealed that there would be a low probability of extremely high exposures to proteins expressed in kernels.

A sensitivity analysis using Crystal Ball determines how much a given input assumption affects the result of the forecast. The percent contribution of protein expression level to total variance ranged from 0.9% (whitetail deer) to 78.4% (slaughter cattle). The contribution of consumption to total variance ranged from 2.3% (whitetail deer) to 97.5% (vole), and the contribution of body weight ranged from 0.3% (quail) to 96.8% (whitetail deer) (Table 3).

The uncertainties associated with our risk assessment can be attributed to few variables. These variables include the toxic endpoint values from each protein, the expression level of each protein in maize, and body weight and consumption values for the ecological receptors. The toxic endpoint values came from a single, deterministic

value for a single species for each protein, and these values were extrapolated for the ecological receptors in our risk assessment. At this time, it is unknown whether any of the NOELs based on dog, mouse, and rat used here can be extrapolated to vole, quail, cattle, or deer. If a 10-fold safety factor is applied to each toxic endpoint, the vole RQ would exceed an RQ of 1.0 for aprotinin. Another uncertainty factor associated with our risk assessment is the use of injected dose values for endpoints in a dietary risk assessment. Because data on variability in protein expression in kernels generally are not publicly available and field-grown plant-based pharmaceuticals are relatively recent, the protein expression estimates in our model used 20% of the mean as a standard deviation for all proteins. This value was based on a few indications in the literature and expert elicitation (K. Wang, Iowa State Univ., personal communication).

The body-weight distribution for whitetail deer came from a combination of values and from these values a triangular distribution was created. To refine the exposure assessment and reduce variance from this input value, more robust measurements of whitetail deer weights would be needed. More data on daily consumption of maize by each receptor also would refine the exposure assessment.

When comparing the RQ values between the proteins, LT-B had values 2.5 orders of magnitude higher when compared to the RQs for gastric lipase, and 1 to 2 orders of magnitude higher when compared to the RQs for aprotinin. The RQ values between species for all proteins at the 90th percentile of exposure showed that the vole RQ was highest. The vole had higher RQs for all proteins because the body weight-to-food consumption ratio is small.

The toxic endpoint value for birds is based on the mouse NOEL. A value specifically for birds would greatly improve the uncertainty of the assessment. The primary area of refinement would be actual consumption rates of maize kernels per day in the presence of other food choices. Lindroth and Batzli (1984) observed that the percent volume of the meadow vole's stomach contained 1–16% seeds in the summer and fall. Lehmann (1984) observed the percent dry volume of crop contents from cultivated grains in the stomachs of bobwhite quails ranged from 3.4–27.4% in the fall and spring. This risk assessment assumed that 100% of the diet came from maize kernels. Clearly, this is not realistic, but is a conservative assumption for the risk assessment.

Except for LT-B, our risk assessment revealed that non-target organism risks to aprotinin and gastric lipase most likely are negligible. Refinements associated with more realistic exposures to LT-B may reduce the RQ less than 1.0. The risk assessment demonstrated that risks will vary between species and between proteins, based primarily on differences in toxic endpoint and consumption rates. It also showed the utility of probabilistic, quantitative risk assessment methodologies, and demonstrates the importance of assessing risks from plant-based pharmaceuticals on a case-by-case basis.

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