Human and Ecological Risk Assessment, 14: 179–193, 2008

ISSN: 1080-7039 print / 1549-7680 online
DOI: 10.1080/10807030701791700

Assessing Risks of Plant-Based Pharmaceuticals: I. Human Dietary Exposure

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

ABSTRACT

Protein-based drugs are the fastest growing class of drugs for the treatment of disease in humans and other animals. However, the current method of producing proteins for pharmaceutical application is predicted to fall short because of population growth and demographic trends. This study characterized human dietary risks using quantitative risk assessment techniques for three pharmaceutical proteins produced in field-grown maize. The three proteins were aprotinin, gastric lipase, and *Escherichia coli* heat-labile enterotoxin B subunit (LT-B). The human dietary risks from the three proteins inadvertently occurring in food were evaluated using three different exposure scenarios so that potential risks could be compared. The three exposure scenarios ranged in conservatism to evaluate the range of risk between the proteins and scenarios. Risk quotients (RQs) were calculated for all three scenarios to integrate exposure and effect (toxicity). The risk assessments revealed that the most conservative scenario produced higher RQs than the other two scenarios. The dietary risks from scenario 1 for aprotinin were three orders of magnitude greater than for scenario 2, and four orders of magnitude greater than for scenario 3. This risk assessment revealed that dietary risks will vary dramatically and depend on factors such as the specific pharmaceutical protein, protein expression, and exposure scenarios. The assessment also reinforced the need for case-by-case assessments.

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

INTRODUCTION

Pharmaceuticals using genetic engineering have been made through protein expression in bacterial, fungal, and mammalian cell cultures. Biotechnology is currently evolving to produce more complex and diverse pharmaceutical proteins in

Received 3 April 2007; revised manuscript accepted 28 June 2007.
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

plants. These pharmaceuticals are known as plant-made or plant-based pharmaceuticals. The plants or 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).

Many people suffer from infectious, inflammatory, and cardiovascular diseases—and these numbers are growing. Protein-based drugs are the fastest growing class of drugs for the treatment of these diseases in humans and other animals. However, the current method of producing proteins for pharmaceutical application is predicted to fall short because of population growth and other demographic trends (Giddings *et al.* 2000).

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). Maize is also free of human and veterinary disease agents. Maize-based vaccines 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 animal models and extensive trials are underway with promising results (Ma *et al.* 2003; Peterson and Arntzen 2004).

Plant-based pharmaceutical production has created public concerns about human-health and ecological risks from potential exposure in the open environment and contamination of the food supply (Peterson and Arntzen 2004; Freese 2005; Kirk *et al.* 2005). The traditional method of vaccine production includes containment in facilities where cell cultures are grown and not exposed to the open environment. With this new method of growing transgenic crops expressing proteins for pharmaceutical production, those proteins will be exposed to the environment. However, in a field there are special containment practices that can be employed to reduce the risk of contamination from the transgenic plants to non-transgenic plants. Risks pertaining to this method of producing pharmaceutical proteins will need to be evaluated to ensure proper precautions are taken before cultivating these plants in the environment.

Pharmaceutical proteins have different structures, stabilities, and toxicities, and, because of this, regulatory guidelines should address each plant and protein combination on a case-by-case basis (Peterson and Arntzen 2004). "The route and frequency of administration should be as close as possible to that proposed for clinical use," but the clinical use of the protein may not result in the same toxicity of indirect exposure through adulterated food consumption (ICH 1997, p. 5). By using a risk-based approach to address these concerns and others, many of the implications can be resolved or managed by understanding where the risks occur in the system.

The objective of this study was to characterize human dietary risks using quantitative risk assessment techniques for three pharmaceutical proteins produced in field-grown maize. The dietary risks from three proteins inadvertently occurring in food were evaluated using exposure scenarios so potential risks could be compared.

MATERIALS AND METHODS

Problem Formulation

Quantitative dietary risk assessments were conducted to characterize the risks associated with inadvertent consumption of pharmaceutical proteins in food as a result of production of those proteins in transgenic maize. The three proteins were aprotinin, gastric lipase, and *Escherichia coli* heat-labile enterotoxin B subunit (LT-B). Three dietary exposure scenarios of varying conservatism were evaluated. Percentiles of dietary exposure and risk were determined for demographic groups such as toddlers, children, and seniors.

Aprotinin Effects Assessment

A complete therapeutic and toxicological review of all three proteins is available in Shama (2006). Aprotinin is a polyvalent protease inhibitor that has been in clinical use since the early 1960s. Manufactured and sold by Bayer as Trasylol®, aprotinin is supplied as a solution containing 10,000 kallikrein inactivation units per milliliter (KIU/ml), which equals 1.4 mg ml^{-1}.

Aprotinin has been shown to decrease protease activity when administered in conjunction with proteins and peptides. The coagulation activity of aprotinin led to the recommendation on the product label to not be used on normally clotting patients due to the risk of thrombosis, but recently this risk has been discounted. In rare first-use cases, aprotinin has caused life threatening anaphylactic reactions, and this risk increases with re-exposure (Bayer Pharmaceuticals Corporation 2003). Currently aprotinin is used intravenously mainly in cardiac surgery for its beneficial effect on the reduction of the perioperative blood loss. It is also referred to as "bovine pancreatic trypsin inhibitor," which affects known serine proteases such as trypsin, chymotrypsin plasmin, and kallikrein.

Aprotinin was traditionally extracted from bovine lungs and is used mainly for medicinal purposes, but also is used in laboratory research and development for controlling degradation of proteins. Some of the medicinal applications include minimization of blood loss during cardiac surgeries to reduce the amount of transfusions needed. It can also be used to reduce blood loss in orthopedic surgeries as well as in pediatric patients undergoing cardiopulmonary bypass surgeries and organ transplantation. Following surgery, aprotinin has been observed to reduce systemic inflammation. Acute pancreatitis is a disease that has benefited from the effects of aprotinin by inhibiting the pancreas from producing enzymes.

Aprotinin Toxic Endpoint

The toxic endpoints (doses that are compared to estimated dietary exposures) for our dietary risk assessment for aprotinin were based on the intravenous dose regime that is administered to patients undergoing cardiopulmonary bypass surgery. The loading dose for patients was chosen as the toxic endpoint over the dog no-observed-effect-level (NOEL) determined by Trautschold *et al.* (1967), because the dog NOEL did not seem as appropriate for a human toxic endpoint. Adverse events have been observed in patients who received the loading dose during surgery, and it is a more conservative value than the dog NOEL. The loading dose was then divided

by the body weight of an average adult male (70 kg), which gave the endpoint value (280 mg aprotinin ÷ 70 kg = 4 mg kg^{-1} body weight (BW)). To our knowledge, there are no publicly available toxicity studies of oral delivery of aprotinin, which would provide a more appropriate toxic endpoint for our assessment.

Gastric Lipase Effects Assessment

Gastric lipase is a protein that was developed as a pharmaceutical to aid in the treatment of exocrine pancreatic insufficiency (EPI), which is an incapacity in the human body to send digestive enzymes to the gut to assimilate food particles (Basque and Ménard 1999). The absence of lipase in the body does not allow digestion of food lipids, which leads to a condition known as steatorrhea, which means there are excess fatty deposits in feces. This condition (EPI) mainly affects cystic fibrosis patients. Lipases are produced naturally in many animals, plants and microbes, and have been consumed for many years without any adverse effects (Coenen *et al.* 1997).

Gastric Lipase Toxic Endpoint

The ingestion NOEL for gastric lipase is 1,000 mg kg^{-1} BW in rats (Coenen *et al.* 1997). Lipases are produced naturally in many animals, plants, and microbes, and the rat NOEL is the only known NOEL for this protein (Coenen *et al.* 1997). Because of its ubiquitous nature, this NOEL may be extrapolated for a human dietary risk. Therefore, the toxic endpoint was based on this value.

E. coli Heat-Labile Enterotoxin B Subunit Effects Assessment

Escherichia coli heat-labile enterotoxin (LT-B) is made up of five "B" subunits and one "A" subunit. The "A" subunit is an active protein that causes adverse effects in the small intestine by entering the epithelial cells and causes water loss from the cells. The "B" subunit is harmless by itself, but will provoke an immune response to the entire enterotoxin. LT is an 84-kilodalton oligomeric protein composed of two major noncovalently linked immunologically distinct peptides called LT-A and LT-B. LT-B is a 55-kilodalton homopentamer of 11.6 kDa peptides responsible for binding of the toxin to the host-cell receptor galactosyl-N-acetylgalactosaminyl-sialyl galactosyl glucosyl ceramide, which is found on the surface of eukaryotic cells (Chikwamba *et al.* 2002).

E. coli Heat-Labile Enterotoxin B Subunit Toxic Endpoint

Beyer *et al.* (2007) found that LT-B at nanogram levels expressed in transgenic maize was immunogenic in mice after oral administration. In a clinical trial in which humans ingested up to 1.1 mg LT-B from transgenic potato, no adverse effects were identified (Tacket *et al.* 1998). With an average adult male human body weight of 70 kg, the endpoint would be 0.016 mg kg^{-1} BW (Wolt *et al.* 2006). Therefore, this endpoint was used for the human dietary risk assessment.

Exposure Assessment

Aprotinin expression in maize

The company ProdiGene began commercial scale-up of aprotinin in field-grown maize in 2003 and 2004. Aprotinin traditionally has been extracted from bovine lungs. ProdiGene's recombinant aprotinin is equivalent to bovine aprotinin (ProdiGene 2004). Zhong *et al.* (1999) found that aprotinin was at a higher concentration in maize embryonic tissue than in endosperm tissue. Azzoni *et al.* (2002) reported the expression level of aprotinin produced from transgenic maize seed to be 0.17%; Zhong *et al.* (1999) observed an expression level of 0.1%.

Gastric lipase expression in maize

Meristem Therapeutics is a company developing a recombinant mammalian gastric lipase. Recombinant gastric lipase is grown in maize and then purified and extracted and used in pre-clinical studies. The mammalian lipase from porcine tissue was selected because it is naturally resistant to digestion by stomach acids and maintains a high enzymatic activity after passage through the stomach. The expression level is approximately 1,000 mg kg^{-1} kernel and there is no expression in other plant organs. The expression was stable over 11 generations (Meristem Therapeutics 2006).

LT-B expression in maize

Chikwamba *et al.* (2002) produced the B subunit of the enterotoxin *E. coli* heatlabile enterotoxin in transgenic maize seed. In their study, the LT-B gene was regulated by a 27-kDa gamma zein promoter, a seed-specific promoter and no LT-B expression was detected in callus tissues. Of 19 LT-B expression P77 events, 11 had LT-B protein levels higher than 0.01% of total aqueous-extractable protein. Two events (P77-2 and P77-3) had LT-B levels of as much as 0.07%.

Chikwamba *et al.* (2003) showed that LT-B protein was detected internally and externally in starch granules of maize. The strong association between the starch granules and the protein give an effective co-purification of the antigen in the processing of the starch fraction of the corn kernels, thermostability, and resistance to peptic degradation in simulated digestion fluids (Chikwamba *et al.* 2003). The strong association would help to understand and monitor for any chance of inadvertent occurrence whereas the thermostability and resistance to peptic degradation indicates the antigen would be stable after ingestion and might be a concern if the LT-B maize is inadvertently mixed with food (Chikwamba *et al.* 2003).

Maize-derived LT-B has been extracted to reveal kernel expression levels of 9.2% of total soluble protein, and 3.7% of total soluble protein has been achieved by using seed specific promoters. As much as 350 mg kg^{-1} LT-B could be expressed in kernels with the seed specific promoters (Chikwamba *et al.* 2002).

Protein expression assumptions

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 1,000 mg kg^{-1}.

Dietary Exposure Scenarios

Scenario 1

The first, and most conservative, exposure scenario for this risk assessment assumed that the transgenic maize expressing the therapeutic protein was harvested and accidentally taken to a specialty food processing facility. The specialty food processing facility makes tortilla chips, and the transgenic maize was made directly into the tortilla chips with no dilution from non-transgenic maize. During the food-making process, the protein was not denatured. The tortilla chips were then eaten by individuals in a variety of age ranges. The expression level of the proteins was calculated by multiplying the weight of a bag of tortilla chips (0.3827 kg) by each expression level. The exposure was calculated by multiplying the estimated protein expression levels by the consumption (assuming consumption of the entire bag of chips in one day for all age groups except toddlers, who we assumed consumed one-half bag) and then dividing that product by the appropriate age-specific body weights (Table 1). This exposure scenario was similar to Wolt *et al.* (2006) in that the transgenic maize was harvested and made directly into tortilla chips with no loss of protein function. However, their scenario assumed a dilution with non-transgenic maize. Also, this risk assessment was conducted on a variety of demographic groups rather than just a high-end consumer group.

Scenario 2

The second exposure scenario assumed that there was a harvest of transgenic maize. The maize was accidentally taken to a food processing facility. However, unlike scenario 1, the maize was diluted with non-transgenic maize, but the protein in the maize does not degrade during processing. This scenario assumed the same dilution factors as Wolt *et al.* (2006). For this scenario it is assumed that 1 ha of transgenic maize will yield 5 Mg (Wang *et al.* unpublished). The 1-ha field was harvested and taken to a dry mill with a daily milling capacity of 2,000 Mg (AgMRC 2003) where the maize was processed into dry-milled food products. Therefore, the percentage of the protein adulterating the maize processed into food products was 0.25% (99.75% dilution). All dry-food products derived from maize (*e.g.*, chips, flour, bran, and starch) from this processing facility were assumed to have the potential to contain transgenic maize and could be eaten by any age group (infant to elderly).

Table 1. Dietary risk assessment results from exposure scenario 1.

Subgroup (age in years)	Aprotinin RQ[a] (100 mg kg^{-1})	Gastric Lipase RQ (1,000 mg kg^{-1})	LT-B RQ (500 mg kg^{-1})
Adults—males; 73.8 kg	0.13	0.01	162.05
Adults—females; 60.6 kg	0.16	0.01	197.35
Youth (10–12); 40.9 kg	0.23	0.01	292.41
Children (5–6); 21.15 kg	0.45	0.02	565.46
Toddlers (2–3); 14.3 kg	0.34	0.02	418.16

[a]RQ = Risk Quotient [dietary exposure ÷ toxic endpoint].

Scenario 3

The third exposure scenario assumed that the edge of a field expressing transgenic, pharmaceutical-maize pollen drifted to a neighboring non-pharmaceutical maize field (kernels had not been formed, fertilization happened when the maize pollen drifted from the transgenic maize field), and the protein was then expressed in 8% of the non-transgenic maize (92% dilution). The 8% expression of transgenic maize in non-transgenic maize was derived from a study conducted by Pla *et al.* (2006). They determined that gene flow frequency was a function of the distance between the donor transgenic maize and non-transgenic maize in fields immediately adjacent to each other (*i.e.*, without a buffer zone).

The assumptions were that the transgenic maize was planted with no distance between itself and the receptor (non-transgenic) field. The normal distance required between receptor (non-transgenic) maize and source maize (transgenic) is 0.8 km to 1.6 km and a 15.24 m fallow zone around the entire source maize field (Christensen *et al.* 2004, USDA APHIS 2006). However, as a reasonable worst-case assumption, this risk assessment does not include any of the required precautions for potential pollen flow. No border rows around the source field site were planted to minimize pollen flow.

The neighboring maize that was assumed to express both transgenic and non-transgenic maize was harvested and taken to a processing facility. The original dilution factor of 99.75% from Scenario 2, and the second dilution factor of 92% from pollen fertilization to the non-transgenic field were used for this scenario. The two dilution factors were multiplied together for a dilution product that was used in the dietary risk model (see later). The "non-transgenic" field was harvested and taken to a processing facility and made into dry-milled maize products. All food products derived from maize (*e.g.*, chips, flour, bran, and starch) from this facility were assumed to have the potential to contain transgenic maize and could be eaten by any age group (infant to elderly).

Demographic groups

Five subgroups were used for Scenario 1: adult males (71.8 kg), adult females (60 kg), youth 10–12 years old (40.9 kg), children 5–6 years old (21.1 kg), and toddlers 2–3 years old (14.3 kg). Body weights were obtained from the U.S. Environmental Protection Agency's (USEPA's) Exposure Factors Handbook (USEPA 1997). Six subgroups were selected for Scenarios 2 and 3: total U.S. population, non-nursing infants, children 1–6 years old, females 13+ (pregnant/not nursing), males 20+ years old, and seniors 55+ years old.

Food consumption assumptions

Acute dietary exposures were defined as a single-day event. The Food Commodity Intake Database (FCID) was used with the Continuing Surveys of Food Intake by Individuals (CSFII) to determine the dietary consumption of individuals through consumption of food products containing maize for Scenarios 2 and 3. The FCID is a database that was developed by the U.S. Department of Agriculture (USDA) for use by the USEPA and other organizations when conducting exposure components of dietary risk assessments. The CSFII was created by the USDA and it measures the

foods actually eaten by individuals. The survey collects data such as household size, income, race, age, sex, and where the food was purchased, how it was prepared, and where it was eaten. The Dietary Exposure Evaluation Model (DEEM) uses the information from the CSFII; therefore, DEEM was used to estimate the inadvertent dietary intake and risk of the therapeutic proteins in food (Kidwell *et al.* 2000).

Scenario 1 did not use DEEM because only one food item (tortilla chips) was included in the exposure estimate and DEEM cannot calculate dietary exposures based on one single, branded food item. Scenario 1 assumed that each individual consumed an entire bag of tortilla chips in one day, except for toddlers. For Scenarios 2 and 3, data on body weights and consumption patterns for these demographic groups were part of the algorithms within the DEEM software. DEEM calculated dietary exposures to each protein by multiplying the protein expression level (100, 500, or 1,000 mg kg^{-1}) in maize kernels by the dilution factors and by records of consumption of food products containing maize. Then, DEEM determined percentiles of dietary exposure to the protein within each demographic subgroup.

Risk Characterization

Risk quotients (RQs) were calculated for all three scenarios to integrate exposure and effect (toxicity). The dietary exposure values were determined probabilistically within DEEM. The toxic endpoint and level of expression were fixed (*i.e.*, deterministic). 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

Scenario 1

In this worst-case scenario with deterministic protein residue and body weight estimates, it was assumed that each individual consumed an entire bag of tortilla chips in one day, regardless of body weight. The lowest RQ with aprotinin expression at 100 ppm was for adult males (0.13) and the highest was for toddlers (0.34) (Table 1). Gastric lipase RQs for Scenario 1 did not exceed an RQ of 1.0 for 1,000 mg kg^{-1} expression. The RQ values ranged from 0.01 to 0.02 (Table 1). LT-B, unlike aprotinin and gastric lipase, had RQs greater than 1.0. The RQ values ranged from 162.05 to 418.16 (Table 1).

Scenario 2

Scenario 2 did not result in RQs greater than 1.0 for aprotinin or gastric lipase. The range of RQs for aprotinin expression at the 50th percentile of dietary exposure was 1×10^{-6} to 1×10^{-5}, at the 95th percentile the range was 2×10^{-4} to 3×10^{-5}, and at the 99th percentile the range was 6×10^{-5} to 2×10^{-4} (Table 2). The RQ range for LT-B expression at the 50th percentile was 0 to 0.27, at the 95th percentile the range was 1.59 to 4.59, and at the 99th percentile the range was 3.07 to 10.15 (Table 3). The range of RQs for gastric lipase expression at the 50th percentile was 4×10^{-8} to 5×10^{-7}, at the 95th percentile the range was 1×10^{-6} to 7×10^{-6}, and

Table 2. Aprotinin dietary risk assessment results from exposure scenario 2.

	50th percentile		95th percentile		99th percentile	
	Exposure (mg/kg BW)	RQ[a]	Exposure (mg/kg BW)	RQ	Exposure (mg/kg BW)	RQ
U.S. population	0.000007	2×10^{-6}	0.0003	7×10^{-5}	0.0006	2×10^{-4}
Infants[b]	0	0	0.0007	2×10^{-4}	0.001	3×10^{-4}
Children[c]	0.00005	1×10^{-5}	0.0006	2×10^{-4}	0.001	3×10^{-4}
Females 13+[d]	0.000007	2×10^{-6}	0.0002	6×10^{-5}	0.0003	7×10^{-5}
Males 20+	0.000004	1×10^{-6}	0.0002	5×10^{-5}	0.0004	1×10^{-4}
Seniors 55+	0.000004	1×10^{-6}	0.0001	4×10^{-5}	0.0002	6×10^{-5}

[a]RQ = Risk Quotient [dietary exposure ÷ toxic endpoint]; [b]non-nursing (<1 yr. old); [c]1–6 yr. old; [d]pregnant not nursing.

at the 99th percentile the range was 2×10^{-6} to 1×10^{-5} (Table 4). The dilution factor reduced the dietary exposure of the therapeutic proteins and this can be seen in the RQ values in Tables 2–4.

Scenario 3

Scenario 3 did not result in RQs greater than 1.0 for any of the three proteins. The RQ range for aprotinin at the 50th percentile was 0 to 3×10^{-7}, at the 95th percentile the range was 3×10^{-6} to 1×10^{-5}, and at the 99th percentile the range was 5×10^{-6} to 3×10^{-5} (Table 5). The range of RQs for LT-B at the 50th percentile was 0 to 0.01, at the 95th percentile the range was 0.13 to 0.37, and at the 99th percentile the range was 0.25 to 0.81 (Table 6). The RQ range for gastric lipase at the 50th percentile was 0 to 4×10^{-8}, at the 95th percentile the range was 1×10^{-7} to 5×10^{-7}, and at the 99th percentile the range was 2×10^{-7} to 1×10^{-6} (Table 7).

The dietary risks from Scenario 1 for aprotinin were three orders of magnitude greater than for Scenario 2, and four orders of magnitude greater than for Scenario 3. The dietary risks from Scenario 1 for LT-B were one order of magnitude greater than for Scenario 2, and two orders of magnitude greater than Scenario 3. The

Table 3. LT-B dietary risk assessment results from exposure scenario 2.

	50th percentile		95th percentile		99th percentile	
	Exposure (mg/kg BW)	RQ[a]	Exposure (mg/kg BW)	RQ	Exposure (mg/kg BW)	RQ
U.S. population	0.002	0.12	0.0652	4.08	0.1332	8.33
Infants[b]	0	0	0.0254	1.59	0.0491	3.07
Children[c]	0.004	0.27	0.0527	3.29	0.0907	5.67
Females 13+[d]	0.002	0.14	0.0699	4.37	0.1098	6.86
Males 20+	0.002	0.11	0.0735	4.59	0.1624	10.15
Seniors 55+	0.002	0.09	0.0511	3.19	0.0874	5.46

[a]RQ = Risk Quotient [dietary exposure ÷ toxic endpoint]; [b]non-nursing (<1 yr. old); [c]1–6 yr. old; [d]pregnant not nursing.

Table 4. Gastric lipase dietary risk assessment results from exposure scenario 2.

	50th percentile		95th percentile		99th percentile	
	Exposure (mg/kg BW)	RQ[a]	Exposure (mg/kg BW)	RQ	Exposure (mg/kg BW)	RQ
U.S. population	0.00007	7×10^{-8}	0.003	3×10^{-6}	0.006	6×10^{-6}
Infants[b]	0	0	0.007	7×10^{-6}	0.01	1×10^{-5}
Children[c]	0.0005	5×10^{-7}	0.006	6×10^{-6}	0.011	1×10^{-5}
Females 13+[d]	0.00007	7×10^{-8}	0.002	2×10^{-6}	0.003	3×10^{-6}
Males 20+	0.00004	4×10^{-8}	0.002	2×10^{-6}	0.004	4×10^{-6}
Seniors 55+	0.00004	4×10^{-8}	0.001	1×10^{-6}	0.002	2×10^{-6}

[a]RQ = Risk Quotient [dietary exposure ÷ toxic endpoint]; [b]non-nursing (<1 yr. old); [c]1–6 yr. old; [d]pregnant not nursing.

dietary risk from Scenario 1 for gastric lipase was three orders of magnitude greater for Scenario 2, and four orders of magnitude greater than for Scenario 3. Scenario 1 did not have a dilution factor applied to it; therefore, the individuals were consuming the highest amount of protein possible when the protein was expressed in maize and made directly into the single food product, tortilla chips.

The RQs decreased by at least three orders of magnitude in Scenarios 2 and 3 compared to Scenario 1 because of the dilution of the transgenic maize with non-transgenic maize. Scenarios 2 and 3 each had a dilution factor applied to them. Scenario 2's dilution factor came from the mixing of the non-transgenic maize with the transgenic maize. Scenario 3's dilution factor came from transgenic-maize pollen fertilizing the non-transgenic maize and then harvesting the non-transgenic maize (partially transgenic) and mixing it with other non-transgenic maize. Although still using highly conservative assumptions, each of these more refined dietary exposure estimates did not exceed toxic endpoints, even at the 99th percentile of exposure.

An adult male would have to consume about 2,800 g per day of aprotinin maize expressed at 100 mg kg^{-1} (Scenario 1) to reach the toxic endpoint value, which is the same as an RQ value of 1.0. For LT-B expressed at 500 mg kg^{-1} this value would be 2.24 g of maize per day, and for gastric lipase expressed at 1,000 mg kg^{-1}, this value

Table 5. Aprotinin dietary risk assessment results from exposure scenario 3.

	50th percentile		95th percentile		99th percentile	
	Exposure (mg/kg BW)	RQ[a]	Exposure (mg/kg BW)	RQ	Exposure (mg/kg BW)	RQ
U.S. population	0.000001	3×10^{-7}	0.00002	6×10^{-6}	0.00005	1×10^{-5}
Infants[b]	0	0	0.00005	1×10^{-5}	0.0001	3×10^{-5}
Children[c]	0.000004	1×10^{-6}	0.00005	1×10^{-5}	0.00009	2×10^{-5}
Females 13+[d]	0.000001	3×10^{-7}	0.00002	5×10^{-6}	0.00002	6×10^{-6}
Males 20+	0	0	0.00002	4×10^{-6}	0.00004	9×10^{-6}
Seniors 55+	0	0	0.00001	3×10^{-6}	0.00002	5×10^{-6}

[a]RQ = Risk Quotient [dietary exposure ÷ toxic endpoint]; [b]non-nursing (<1 yr. old); [c]1–6 yr. old; [d]pregnant not nursing.

Table 6. LT-B dietary risk assessment results from exposure scenario 3.

	50th percentile		95th percentile		99th percentile	
	Exposure (mg/kg BW)	RQ[a]	Exposure (mg/kg BW)	RQ	Exposure (mg/kg BW)	RQ
U.S. population	0.0002	0.01	0.005	0.326	0.011	0.67
Infants[b]	0	0	0.002	0.127	0.004	0.25
Children[c]	0.0003	0.02	0.004	0.263	0.007	0.45
Females 13+[d]	0.0002	0.01	0.006	0.35	0.009	0.55
Males 20+	0.0001	0.01	0.006	0.367	0.013	0.81
Seniors 55+	0.0001	0.01	0.004	0.255	0.007	0.44

[a]RQ = Risk Quotient [dietary exposure ÷ toxic endpoint]; [b]non-nursing (<1 yr. old); [c]1–6 yr. old; [d]pregnant not nursing.

would be 70,000 g of maize per day. For Scenario 2, an adult male would have to consume 1,120 kg of aprotinin maize, 896 g of LT-B maize, and 28,000 kg of gastric lipase per day to reach each protein's respective toxic endpoint. Because of further dilution due to pollen flow in Scenario 3, an adult male would have to consume 14,000 kg of aprotinin maize, 11.2 kg of LT-B maize, and 3,500,000 kg of gastric lipase maize per day to reach each protein's respective toxic endpoint.

Wolt *et al.* (2006) assessed the risk of unintended antigen occurrence in food by establishing scenarios using maize-expressed LT-B as a case study. The scenarios in our risk assessment are similar to the scenarios in Wolt *et al.* (2006), except in our scenario we considered additional age groups in which individuals consumed tortilla chips, whereas they assumed a high-end consumer group (13- to 19-year-old males) consuming three servings of tortilla chips made solely from maize expressing LT-B. This is a worst-case scenario similar to Scenario 1 of this risk assessment. Wolt *et al.* (2006) considered dilution scenarios in which the LT-B maize was mixed with non-transgenic maize and made into tortilla chips and then consumed by high-end consumers. Scenario 2 of this risk assessment used their dilution factors, but we then

Table 7. Gastric lipase dietary risk assessment results from exposure scenario 3.

	50th percentile		95th percentile		99th percentile	
	Exposure (mg/kg BW)	RQ[a]	Exposure (mg/kg BW)	RQ	Exposure (mg/kg BW)	RQ
U.S. population	0.000005	5×10^{-9}	0.0002	2×10^{-7}	0.0005	5×10^{-7}
Infants[b]	0	0	0.0005	5×10^{-7}	0.001	1×10^{-6}
Children[c]	0.000043	4×10^{-8}	0.0005	5×10^{-7}	0.0009	9×10^{-7}
Females 13+[d]	0.000006	6×10^{-9}	0.0002	2×10^{-7}	0.0002	2×10^{-7}
Males 20+	0.000003	3×10^{-9}	0.0001	1×10^{-7}	0.0004	4×10^{-7}
Seniors 55+	0.000003	3×10^{-9}	0.0001	1×10^{-7}	0.0002	2×10^{-7}

[a]RQ = Risk Quotient [dietary exposure ÷ toxic endpoint]; [b]non-nursing (<1 yr. old); [c]1–6 yr. old; [d]pregnant not nursing.

assumed that the diluted maize was processed into dry milled corn products (*e.g.*, chips, flour, bran, and starch) as well.

Howard and Donnelly (2004) proposed a quantitative safety assessment model for aprotinin produced in transgenic maize. Similar to our risk assessment, they assumed an expression level of 100 mg kg^{-1} and no inactivation or loss of function of the protein in transgenic maize. However, the endpoint value used in their analysis (125 mg kg^{-1} BW, the dog NOEL after injection) was 31-fold more than our value of 4 mg kg^{-1} BW (loading dose of aprotinin when administered to human patients undergoing cardio-pulmonary bypass surgery). The endpoint of 4 mg kg^{-1} BW is more appropriate because the loading dose is a value that has been determined based on human exposure to aprotinin. This dosage has resulted in deleterious effects in patients and is a more conservative value than the dog NOEL.

Howard and Donnelly (2004) assumed a 70 kg person consumed a 0.45 kg box of cereal with 90% of protein activity lost due to processing (heat inactivation) with protein expression at 100 mg kg^{-1}. Their risk assessment was much different from our risk assessment because they used a different approach to determine the level of risk. They did not assume multiple scenarios, instead assuming a case in which the protein is degraded during processing. This is different from our risk assessment where dilution was considered by inadvertent mixing with non-transgenic maize. They did not use DEEM to determine the amount consumed daily by individuals, but instead determined the amount consumed by using an ingestion rate value based on an inactivation factor (empirically based on the normal preparation of corn grain for food uses). Both our risk assessment and that of Howard and Donnelly (2004) assumed that aprotinin was as active orally as by injection, which currently represents a substantial uncertainty.

The scenarios applied to our risk assessment did not incorporate USDA permit guidelines (USDA APHIS 2006). We assumed worst-case scenarios; therefore, we did not incorporate the USDA permit guidelines. Had those guidelines been taken into account with our risk assessment (*e.g.*, distance between fields, planting time, and fallow zone), exposure and subsequent dietary risks for all scenarios would be much less.

The uncertainties associated with our risk assessment include the expression level of the protein in maize and the endpoint values for aprotinin and LT-B. Gastric lipase has an experimentally derived NOEL that most likely can be used for risk assessment. The endpoint value used for aprotinin came from the intravenous loading dose that is administered to patients undergoing cardio-pulmonary bypass surgery. This dosage has resulted in deleterious effects in patients, but it also is administered via intravenous injection, not oral administration. Therefore, the toxic endpoint based on this value is highly uncertain and may not be acceptable for risk assessment. The endpoint value for LT-B is from a study in humans and represents a dosage in which no effects were observed, but that study did not experimentally derive a NOEL. Consequently, for aprotinin and LT-B, the dietary risk values presented here should be cautiously interpreted.

Because differences in toxic endpoints were responsible for the greatest differences in dietary risk, more precise knowledge is needed regarding toxicity of these pharmaceutical proteins as it relates to oral ingestion via adventitious presence in food. Typically, NOELs are not established for pharmaceuticals. However, because of the possibility of production of pharmaceutical proteins in crop species and

unintended occurrence in food, NOELs should be established. Further, the NOELs should be determined for the appropriate route of exposure—oral ingestion. For aprotinin, there is no publicly available toxicity information for the oral route of exposure. Determination of an acute, oral NOEL would substantially reduce uncertainty associated with dietary risk of that protein.

Another uncertainty factor is allergenicity. Allergic reactions have been observed after re-exposure to high-dose aprotinin. Aprotinin is immunogenic and induces immunoglobulin G and E (IgG and IgE) responses, but usually only after a secondary exposure (Beierlein *et al.* 2000; Milano *et al.* 2002). It is possible for aprotinin to cause adverse effects after repeated applications based on its antigenic properties and a prick test is recommended before administration of aprotinin.

Our risk assessment demonstrates that human dietary exposures and risks will vary dramatically and depend on factors such as the specific pharmaceutical protein, protein expression, and exposure scenario. Previously, Nap *et al.* (2003), Peterson and Arntzen (2004), Kirk *et al.* (2005), and Ma *et al.* (2005) argued for the need to assess risks from plant-based pharmaceuticals on a case-by-case basis. The assessment presented here, using three different pharmaceutical proteins, supports that argument.

ACKNOWLEDGMENTS

We thank J. Wolt (Iowa State University) for reviewing a previous version of this article. This study was funded by a grant from the Biobased Institute at Montana State University and the Montana Agricultural Experiment Station.

REFERENCES

AgMRC (Agricultural Marketing Resource Center). 2003. The U.S. Corn Masa Industry: Structure and Implications for the Great Plains Region. Prepared for AgMARC by Sparks Companies, Inc., Department of Agricultural Economics, Kansas State University, Manhattan, Kansas, USA

Azzoni AR, Kusnaki AR, Miranda EA, *et al.* 2002. Recombinant aprotinin produced in transgenic corn seed: Extraction and purification studies. Biotechnol Bioeng 80:268–76

Basque J-R and Ménard D. 1999. Establishment of culture systems of human gastric epithelium for the study of pepsinogen and gastric lipase synthesis and secretion. Microscopy Res Techn 48:293–302

Bayer Pharmaceuticals Corporation. 2003. Trasylol® Aprotinin Injection. Product Label. West Haven, CT, USA

Beierlein W, Scheule AM, Antoniadis G, *et al.* 2000. An immediate, allergic skin reaction to aprotinin after reexposure to fibrin sealant. Transfusion 40:302–05

Beyer AJ, Wang K, Umble AN, *et al.* 2007. Low-dose exposure and immunogenicity of transgenic maize expressing the *Escherichia coli* heat-labile toxin B subunit. Environ Health Perspect 115:354–60

Chikwamba R, McMurray J, Shou H, *et al.* 2002. Expression of a synthetic *E. coli* heat-labile enterotoxin B sub-unit (LT-B) in maize. Mol Breeding 10:253–65

Chikwamba RK, Scott MP, Mejia LB, *et al.* 2003. Localization of a bacterial protein in starch granules of transgenic maize kernels. Proceed National Acad Sci 100:11127–32

Christensen PJ, Misra MK, Rai S, *et al.* 2004. Confined Production Processes for Non-food Corn. Biosafety Institute for Genetically Modified Agricultural Products, Iowa State University, Ames, IA, USA

Coenen TMM, Aughton P, and Verhagen H. 1997. Safety evaluation of lipase derived from *Rhizopus oryzae*: Summary of toxicological data. Food Chem Toxicol 35:315–22

Fischer R and Emans N. 2000. Molecular farming of pharmaceutical proteins. Transgenic Res 9:279–99

Freese B. 2005. Biopharming: Case Study of Corn-Produced Aprotinin. Friends of the Earth, for Genetically Engineered Food Alert. http://www.foe.org/biopharm/

Giddings G, Allison G, Brooks D, *et al.* 2000. Transgenic plants as factories for biopharmaceuticals. Nature Biotechnol 18:1151–55

Howard JA and Donnelly KC. 2004. A quantitative safety assessment model for transgenic protein products produced in agricultural crops. J Agricul Environ Ethics 17:545–58

ICH (International Conference on Harmonisation). 1997. Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals, ICH Harmonised Tripartite Guideline S6, 16 July 1997. ICH Secretariat, Geneva, Switzerland

Kidwell JL, Tomerline JR, and Petersen BJ. 2000. DEEM™ Dietary Exposure Evaluation Model. Novigen Sciences, Inc. Washington, DC, USA

Kirk DD, McIntosh K, Walmsley AM, *et al.* 2005. Risk analysis for plant-made vaccines. Transgenic Res 14:449–62

Ma JK-C, Drake PMW, and Christou P. 2003. The production of recombinant pharmaceutical proteins in plants. Nature Reviews Genetics 4:794–805

Ma JK-C, Barros E, Bock R, *et al.* 2005. Molecular farming for new drugs and vaccines: Current perspectives on the production of pharmaceuticals in transgenic plants. EMBO Reports 6:593–99

Meristem Therapeutics. 2006. Merispase® Product Label. Clermont-Ferrand, France. http://www.meristem-therapeutics.com/sommaire_en.php3

Milano CA, Patel VS, Smith PK, *et al.* 2002. Risk of anaphylaxis from aprotinin re-exposure during LVAD removal and heart transplantation. J Heart Lung Transplant 21:1127–30

Nap J-D, Metz PLJ, Escaler M, *et al.* 2003. The release of genetically modified crops into the environment: Part 1. Overview of current status and regulations. The Plant Journal 33:1–18

Peterson RKD and Arntzen CJ. 2004. On risk and plant-based biopharmaceuticals. Trends in Biotechnol 22:64–6

Pla M, Messeguer J, and Mele E. 2006. Gene flow from GE to conventional maize using real-time PCR. ISB News Report. August

Prodigene. 2004. AproliZean TM—Recombinant Aprotinin from Maize, Version I, 3/2004. www.prodigene.com

Shama LM. 2006. Risk Assessment of Plant-Based Pharmaceuticals and Biologics. Masters Thesis. Montana State University, Bozeman, MT, USA

Shama LM and Peterson RKD. 2004. The benefits and risks of producing pharmaceutical proteins in plants. Risk Management Matters 2:28–33

Tacket CO, Mason HS, Losonsky G, *et al.* 1998. Immunogenicity in humans of a recombinant bacterial antigen delivered in transgenic potato. Nature Med 4:607–09

Trautschold I, Werle E, and Zickgraf-kudel G. 1967. Trasylol. Biochem Pharmacol 16:59–72

USDA APHIS (US Department of Agriculture Animal and Plant Health Inspection Service). 2006. Draft Guidance for APHIS Permits for Field Testing or Movement of Organisms with Pharmaceutical or Industrial Intent. Washington, DC, USA. http://www.aphis.usda.gov/brs/pdf/Pharma_Guidance.pdf

USEPA (U.S. Environmental Protection Agency). 1997. Exposure Factors Handbook Volume 1—General Factors. EPA/600/P-95/002Fa. Washington, DC, USA

Wang K. 2006. Personal communication. Iowa State University, Ames, Iowa, USA

Wolt JD, Wang K, and Peterson RKD. 2006. Assessing risk of unintended antigen occurrence in food: A case instance for maize-expressed LT-B. Hum Ecol Risk Assess 12:856–70

Zhong GY, Peterson D, Delaney DE, *et al.* 1999. Commercial production of aprotinin in transgenic maize seeds. Molecular Breeding 5:345–56