11) Prueksaritanont, T., Lin, J. H., Baillie, T. A.: *Toxicol. Appl. Pharmacol.*, **217**(2), 143-152 (2006).
12) 非臨床薬物動態試験ガイドライン（平成10年6月26日医薬審第496号）.
13) Guidance for Industry, Drug Interaction Studies－Study Design, Data Analysis, and Implications for Dosing and Labeling, FDA CDER Draft Guidance, Published in Federal Register, September (2006).
14) 第21回日本薬物動態学会年会 講演要旨集, 171-177 (11月29日～12月1日, 2006, 東京).
15) 医薬品評価フォーラム キックオフミーティング－画期的新薬を目指して：基礎から臨床へ－. 講演要旨集, 社団法人日本薬学会 (1月13日, 2007, 共立薬科大学).
16) 山田久陽, 山口順一, 飯田 泉, 奥山 茂：日薬理誌, **127**, 473-480 (2006).

The Journal of Toxicological Sciences,
Vol.32, No.4, 329-341, 2007

– Review –

CURRENT OPINION: SAFETY EVALUATION OF DRUG METABOLITES IN DEVELOPMENT OF PHARMACEUTICALS

Shinsaku NAITO[1], Shigeru FURUTA[1], Takemi YOSHIDA[2], Mitsukazu KITADA[3], Osamu FUEKI[4], Takashi UNNO[5], Yasuo OHNO[6], Hiroshi ONODERA[4], Nobuyuki KAWAMURA[7], Misao KUROKAWA[1], Fumio SAGAMI[1], Kazutoshi SHINODA[4], Takahiro NAKAZAWA[1] and Tsuneyoshi YAMAZAKI[8]

[1]*Japan Pharmaceutical Manufacturers Association, Drug Evaluation Committee, Non-clinical Evaluation Subcommittee, Torii Nihonbashi Bldg., 3-4-1 Nihonbashi-Honcho, Chuo-ku, Tokyo 103-0023, Japan*

[2]*Showa University, School of Pharmaceutical Sciences, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan*

[3]*Chiba University Hospital, 1-8-1 Inohana, Chuo-ku, Chiba 260-8677, Japan*

[4]*Pharmaceuticals and Medical Devices Agency, Shin-Kasumigaseki Building, 3-3-2 Kasumigaseki, Chiyoda-ku, Tokyo 100-0013, Japan*

[5]*Pharmaceutical Research and Manufactures of America, 4F Landic II, Toranomon, Minato-ku, Tokyo 105-0001, Japan*

[6]*National Institute of Health Sciences, 1-18-1 Kamiyoga Setagaya-ku, Tokyo 158-8501, Japan*

[7]*European Federation of Pharmaceutical Industries and Associations Japan, Technical Committee, GSK Bldg., 4-6-15 Sendagaya, Shibuya-ku, Tokyo 151-8566, Japan*

[8]*Kyoritsu University of Pharmacy, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512, Japan*

(Received June 19, 2007; Accepted August 2, 2007)

ABSTRACT — Safety assessment of drug metabolites in the development of pharmaceuticals was discussed in January 2007 at the kick-off meeting of a "Drug Evaluation Forum", with reference to the views of clinicians and other academic representatives. Safety evaluation of metabolites cannot readily be based on a single theoretical framework, and basically a case-by-case approach is called for. These evaluations should be performed precisely and an accurate profile secured taking into account adverse reactions that are unpredictable from the parent compound administered in clinical studies and any signs or symptoms that may be associated with the metabolites. In addition, elimination of scientifically meaningless metabolite safety assessment studies is essential for prompt supply of high-quality drugs to the medical frontline. Preparation of an outline concept paper would be useful for achievement of shared understanding of issues of this type. Collective viewpoints obtained in this fashion are also relevant to the discussion on the need for guidance, and given a degree of flexibility may also be helpful for drug development and, in turn, society at large.

KEY WORDS: Drug Development, Metabolites, Active metabolites, Reactive metabolites, Safety, Non-Clinical Study, Clinical Study

INTRODUCTION

Information on pharmacokinetics in laboratory animals and humans obtained in the course of drug development is essential for clarifying *in vivo* toxicity mechanisms or pharmacological effects, as well as for determining appropriate formulations and methods of administration. However, ethnic differences and individual variations in pharmacokinetics exist, and drug-metabolizing activity is affected by diverse factors. Comparative pharmacokinetic studies of animals and humans are useful for extrapolating the results of toxic-

Correspondence: Shinsaku NAITO (E-mail: naitousn@otsukakj.co.jp)

ity and pharmacological studies to humans and for determining drug efficacy and safety, as well as for predicting drug interactions when assessing individual variations in pharmacological effects or consideration to patients with hypersensitive reactions. However, while the results of toxicity studies and pharmacological studies in animals with pharmacokinetics similar to those of humans are valuable for extrapolating the results of animal experiments to humans, care is required in interpreting the results obtained in animal species when the metabolites differ significantly from those in humans. Discussion of safety assessment of metabolites is essential, and to this end this article aims to distil the viewpoints on this subject in order to promote understanding of the research undertaken by companies involved in drug development, the drug approval process, and the medical frontline. Such discussion should be useful for drafting of guidelines. However, this article is only a compilation of viewpoints on current issues, and it goes without saying that these should be updated as the science progresses.

The need to study the toxicity and pharmacological effects of major metabolites for safety assessment of metabolites in drug development was outlined in Japan in a notification from the Ministry of Health and Welfare in 1975 (Yakushin No. 526, 1975). However, this notification did not go so far as to indicate the specific studies required for safety assessment, but in general, metabolites have been assessed in single-dose toxicity studies. Since this notification can also be interpreted as requiring the assessment of all metabolites, at the ICH-1 held in 1991 the Japanese authorities suggested that toxicological assessment of metabolites is necessary when the metabolite is present in humans, but not found in animal species used in toxicity studies, when the metabolite is formed in particularly large amounts in humans, and also when the pharmacologic and toxicologic activity of the metabolite is considered significant (Ohno, 1992). However, the amount of metabolites formed that would necessitate safety assessment was not clearly defined. Such assessments in excess of the minimum have been made on a case-by-case basis by the tripartite authorities in the US, EU and Japan. The US Food and Drug Administration (FDA) and Pharmaceutical Research and Manufacturers of America (PhRMA) have since discussed the degree of metabolite formation requiring safety assessment (Baillie *et al.*, 2002, 2003; Hastings *et al.*, 2003). With reference to this background, the toxicity representative of the Pharmaceuticals and Medical Devices Evaluation Center in Japan presented views on the toxicological assessment of unique human metabolites from a personal standpoint (Toxicology Q&A: Question 16; Toxicological evaluation of unique human metabolites, 2003). In addition, a questionnaire survey of the members of the Japan Pharmaceutical Manufacturers Association (JPMA) found that toxicity studies of metabolites are a matter of great concern (Inoue *et al.*, 2005). Further, the FDA released in June 2005 a draft guidance concerning their views on safety testing of metabolites found in clinical studies (FDA CDER, Guidance for Industry, SafetyTesting of Drug Metabolites (Draft). 2005).

In the draft guidance, the FDA provided definitions of terms such as unique human metabolites, pharmacologically active metabolites, and major metabolites. They also provided viewpoints on when it is deemed necessary to conduct a safety assessment of metabolites. Circumstances when the safety of metabolites should be considered included when the metabolites are unique to humans; or when they are formed in greater amounts in humans than in animals; or when they exceed 10 percent of (the lower of) the dosage or systemic exposure. The guidance also established the timing and method of performance of such assessments. The four types of metabolite toxicity studies requested by the FDA are primarily: (1) general toxicity studies with direct administration of metabolites (14- ~ 90-day dosing including toxicokinetic (TK) studies, with a modified route of administration); (2) the minimum necessary genotoxicity studies; (3) performance of studies on embryonic-fetal development; and (4) carcinogenicity studies when deemed necessary based on the results of the targeted indication or results from other studies. In addition, the FDA recommends that when metabolite exposure has been inadequate in studies such that safety has not been fully assessed in studies already conducted, a synthesized reference standard should be prepared and the safety of the metabolite assessed using a route of administration whereby adequate exposure can be obtained in an appropriate animal species. The results of these studies should be reported to the FDA prior to the commencement of Phase III clinical studies. The JPMA has submitted public comments on this draft guidance after compiling the views of its members. In particular, the JPMA has pointed to the inadequately clear rationale for the criterion for a major metabolite as being 10 percent of the dose or systemic exposure of the parent. JMPA has also commented on such matters as the cases presented in which the major metabolites in blood and urine differ or in which they do not reflect exposure at

the target site of toxicity, on cases of dosage discrepancies, and also on cases in which manifestations of toxicity are ascribable to the C_{max} or AUC. However, as of January 2007, the FDA draft guidance has not been finalized.

The guidance from the European Medicines Agency (EMEA) on drug interactions (CPMP/EWP/560/95, 1997) recommends that *in vitro* and/or *in vivo* drug interaction studies be conducted for metabolic pathways responsible for 30% or more of total clearance, and that studies may be required even for metabolites with lower levels of exposure that demonstrate toxicity or pharmacological activity.

At present, viewpoints on handling of metabolites vary among reference papers (Baillie *et al.*, 2002, 2003; Hastings *et al.*, 2003; Smith and Obach, 2005, 2006; Davis-Bruno and Atrakchi, 2006; Guengerich, 2006; Prueksaritanont *et al.*, 2006) and guidance documents from different countries concerning the safety assessment of metabolites (CPMP/EWP/560/95, 1997; Iyakushin No. 496, 1998; FDA CDER, Guidance for Industry, Bioavailability and Bioequivalence Studies for Orally Administered Drug Products - General Considerations, 2003, Safety Testing of Drug Metabolites (Draft), 2005, Drug Interaction Studies – Study Design, Data Analysis, and Implications for Dosing and Labeling (Draft), 2006). Opinions are divided on what the criteria should be triggering toxicity studies with metabolites. In Japan, as well, the FDA draft guidance has prompted consideration of the standards for safety assessment of major metabolites. Metabolite studies were discussed at Forum 2006 of the 21st Annual Meeting of the Japanese Society for the Study of Xenobiotics held in November 2006 (JSSX Annual Meeting Abstracts, 2006) with emphasis on collaboration between pharmacokinetic and toxicity scientists. The importance of the role played by regulatory sciences in drug development was recognized in such discussions. At the same time in early 2007 the Division of Regulatory Sciences of the Pharmaceutical Society of Japan established a "Drug Evaluation Forum" bringing together industry, government, and university representatives. Academic participation in the discussions allowed pharmaceutical companies and representatives of the Pharmaceuticals and Medical Devices Agency to discuss practical issues while maintaining neutrality and impartiality concerning the process of new drug approval. A significant industry-government-university discussion on the theme of the safety assessment of metabolites took place in January 2007 at the kick-off meeting of this Drug Evaluation Forum. The viewpoints and issues regarding future safety testing of metabolites have now been compiled and are presented in this manuscript. The opinions were gleaned from the discussion at this kick-off meeting concerning the significance and method of metabolite safety assessment with a view to promoting the safety of drugs and identifying their effects in living subjects.

DRUG METABOLISM AND MANIFESTATIONS OF TOXICITY

Drug-metabolizing enzymes and problems associated with metabolites

In addition to exhibiting various effects, drugs administered to living subjects are in many cases excreted after being metabolized in the liver and other organs by enzymes associated with drug metabolism. There are ethnic, individual, gender, and other types of variation in drug-metabolizing enzymes associated with genetic factors, environmental factors and other factors such as clinical condition, concomitant medications, diet, and individual variability.

These considerations reveal major challenges to the safety assessment of metabolites in humans. Moreover, some adverse reactions or adverse effects occurring in humans are associated with the production of "reactive metabolites". However, since reactive intermediates are readily metabolized and may be present only as intermediates in the process of metabolism, it is often difficult to detect reactive metabolites and to supply stable synthesized reference standards. Therefore, in light of the factors exhibiting inter-individual variability referred to above, there are major difficulties with demonstrating the presence of reactive metabolites and with linking them to the mechanisms or manifestations of toxicity. In addition, although the safety of metabolites can in principle be assessed from animal experiments using a parent compound, there are cases in which quantitative or rate-related species differences exists in process of metabolism, or in which unique human metabolites and pharmacologically active metabolites (particularly reactive metabolites) are formed. These cases present significant challenges in assessing the safety of metabolites.

Although the substrate specificity of the CYP1, CYP3, and CYP4 families is relatively well-preserved in both humans and laboratory animals, the CYP2 family exhibits species differences due to its poor substrate correlation (Table 1). In addition, genetic polymorphisms are known to exist among drug-metabolizing enzymes, and effects such as the regulation of enzyme

induction or inhibition by exogenous or endogenous substances have also been identified. Furthermore, it is believed that some of these enzymes are affected by clinical conditions or diseases, concomitant medications, age, smoking, diet, and other circumstances of daily life.

Metabolic activation of drugs and manifestations of toxicity

Examples of metabolic reactions and metabolite formations that may lead to manifestations of toxicity are given in Table 2. In metabolic activation, toxicity is manifested as a result of radical formation (active oxygens, active nitrogens), modification of ribonucleic acids (mutagenicity, carcinogenicity), enzyme inhibition and induction, oxidative phosphorylation, electron transport chain inhibition, inhibition of the liberation or uptake of neurotransmitters (binding with transporters etc.), binding with or modification of receptors (receptor function changes), inhibition or acceleration of ion channels, and binding with or modification of proteins, lipids, and other components of living organisms. In addition, the formation of reactive metabolites (pharmacologically active metabolites linked to manifestations of toxicity) is involved in some adverse reactions, presenting a major challenge to the safety assessment of metabolites (Fig. 1). For example, *N*-acetyl-*p*-benzoquinone-imine, a pharmacologically active metabolite of acetaminophen, has been inferred to cause liver damage through allylation of mitochondrial proteins and other effects. Troglitazone, which has a chroman ring, may also cause liver damage through the formation of semi-quinone radical-mediated quinones and quinone methides in the process of oxidation reactions, as well as through the formation of α-ketoisocyanate by an oxidation reaction at the thiazolidinedione ring site (Yamata *et al.*, 2006). In cases in which glutathione conjugates and acyl glucuronide conjugates are detected as metabolites, concerns exist regarding the possibility of reactive metabolite formation, since there have been cases in which manifestations of toxicity such as serious organ damage were triggered by highly reactive metabolites. Many of the drugs for which concerns exist regarding the possibility of relationships between reactive metabolites and liver damage have not been approved in the US, and of the drugs deemed to have reactive intermediates, five have had approval withdrawn or restrictions on use imposed, and another eight have had black box warnings added to their labels (Table 3). However, given the difficulty of assessing the safety of reactive metabolites despite the conduction of toxicity studies using the glutathione conjugate, acyl glucuronide conjugate, or other final metabolites, there is at present no clear basis for ascribing adverse events induced by such drugs to reactive metabolites. Given the difficulty in detecting reactive metabolite-induced adverse events in clinical studies, it is preferable to reduce the human risks associated with the development of compounds by screening for metabolic stability and pharmacologically active metabolites at the exploratory stage of drug development, and minimizing the development of compounds with reactive metabolites that may cause concern.

In a 2006 questionnaire survey sponsored by the Japanese Society for the Study of Xenobiotics, it was revealed that many pharmaceutical companies are making efforts to detect reactive metabolites through screening early to avoid developing compounds that could cause reactions associated with toxic effects of reactive metabolites. For example, companies perform experiments assessing time-dependent enzyme inhibition reactions; covalent binding to tissue, liver microsomal covalent binding, and GSH adduct formation. Such efforts at screening are expected to reduce

Table 1. Classification of Cytochrome P450 Drug-Metabolism Enzymes.

CYP1 Family:	Two subfamilies (CYP1A1, CYP1A2, and CYP1B1 are expressed in humans): AhR-mediated induction of 1A, involvement in metabolic activation of carcinogens by epoxidation or *N*-hydroxylation, and so on
CYP2 Family:	Many subfamilies (10 types of subfamilies are expressed in humans, including CYP2A, CYP2B, CYP2C, CYP2D, CYP2E, CYP2F, and CYP2J): Major enzymes for metabolism of various drugs with different structures as substrates for CYP2C9, 2C19, 2D6
CYP3 Family:	One subfamily; Induced by steroids and macrolide antibiotics; testosterone 6β- hydroxylation is a prototype: CYP3A4, CYP3A5, and CYP3A7 exist in humans; CYP3A is the most abundant species in human liver
CYP4 Family:	Four subfamilies, catalysis of ω- hydroxylation of fatty acids

the risks associated with development of compounds that form reactive metabolites and that may trigger idiosyncratic drug toxicity (IDT, Yamada *et al.*, 2006).

Clinical problems concerning the manifestation of adverse reactions in pharmacotherapy

Concomitant use of drugs is a daily occurrence in medical practice, and the number of prescriptions given to an individual increases with age as well as the incidence of adverse reactions increases with age. Metabolic profiles vary significantly partly as a result of drug-drug interactions, suggesting that metabolites may also be involved in the manifestation of adverse reactions. Accordingly, while the properties of metabolites constitute essential information for the safe use of drugs, assessment of metabolite toxicity in humans should be based on properties such as the amounts of metabolites formed or their activity.

The results of a meta-analysis published in 1998 by JAMA, (Journal of the American Medical Association; Lazarou *et al.*, 1998), found that serious drug-induced adverse reactions occur in approximately 2.2 million patients per year, and that of these patients as many as 100,000 suffer life-threatening adverse reactions. While the extent to which the toxicity of metabolites is involved in drug-induced adverse reactions is unclear, these reactions are recognized as a major clinical problem. Post-marketing "Dear Doctor Letters" written after the time of approval include reported adverse events occurring after the clinical trial stage and those events not previously identified or described as "severe adverse reactions". In the case of quetiapine fumarate, edaravone, pioglitazone HCl, cephocelis sulfate, and troglitazone, new and severe adverse reac-

Table 2. Metabolic reaction or metabolite production leading to toxicity.

N-hydroxylation (hepatic tumor induction by acetylaminofluorene, hepatotoxicity of acetaminophen, methemoglobinemia by aniline, and some others)
Epoxidation (induction of hepatic tumor or hepatotoxicity by aflatoxin, hepatotoxicity by bromobenzene)
Desulfuration (neurotoxicity by organophosphorus insecticides)
Aldehyde or ketone production (toxicity of ethanol, zomepirac, hexon, etc.)
α, β- Unsaturated aldehyde or ketone production (toxicity of acrolein, benzene, 4-hydroxynonenal, etc.)
Quinone or quinoneimine production (toxicity of acetaminophen or diethylstilbesterol)
Sulfoxide production (hepatotoxicity of thioacetamide)
Acylhalide production (toxicity of halothane or chloroform)
Thionoacylhalide production (renal toxicity of hexachlorobutadiene)
Thioketene production (renal toxicity of hexachlorobutadien)
Radical production (hepatotoxicity of carbon tetrachloride or halothane)
Carbonium ion production (carcinogenesis by nitrosamine compounds, induction of hepatic cancer or hepatotoxicity by cycasin)
Nitronium ion production (carcinogenesis by acetylaminofluorene)
Sulfonium ion production (carcinogenesis by 1, 2-dibromoethane)
Metal ion production (renal toxicity of mercury or cisplatin)

tions not noted during the clinical trial stage were subsequently observed in the post-marketing database. In addition, even when the metabolites themselves lack toxicity, interactions in the processes associated with metabolism can sometimes trigger life-threatening drug interactions, e. g., concomitant administration of 5-FU with sorivudine.

To ensure appropriate use of drugs, it is essential to identify fully both factors associated with the drug such as drug receptors, metabolic enzymes, drug transporters, and those that predispose patients such as genetic factors (gene polymorphisms), environmental

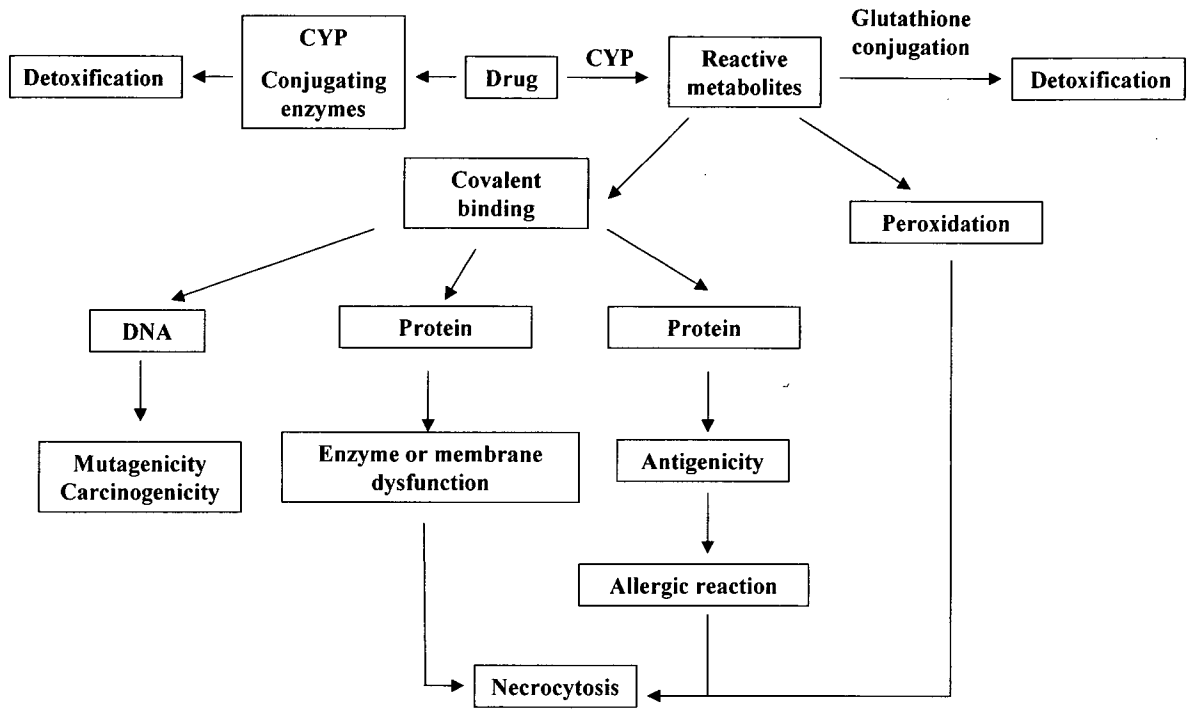

Fig. 1. Metabolic activation and development of toxicity by drugs and chemicals.

Table 3. Hypothesis of Hepatotoxicity of reactive metabolites.

Fourteen drugs with reactive metabolites that have warnings regarding hepatotoxicity
Acetaminophen, Carbamazepine, Clozapine, Diclofenac, Disulfiram, Halothane, Leflunomide, Methyldopa, Rifampin, Tacrin, Tamoxifen, Terbinafine, Ticlopidine, Zileuton
Fourteen drugs with reactive metabolites that have warnings regarding hepatotoxicity & have never been approved in USA
Alpidem, Amineptine, Amodiaquine, Cinchophen, Dihydralazine, Dilevaolo, Ebrotidine, Glafenine, Ibufenac, Isoxanine, Niperotidien, Perhexiline, Pirprofen, Tilbroquinol
Five drugs withdrawn or with restrictions on use due to reactive metabolites
Benoxaprofen, Iproniazid, Nefazodone, Tienilic acid, Troglitazone
Eight drugs with black-box warnings due to reactive metabolites
Dacarbazine, Dantrolene, Felbamate, Flutamide, Isoniazid, Ketoconazole, Tolcapone, Valproic acid

factors, diet, alcohol use, smoking, medical disorders, and concomitant use of medications. The findings of post-marketing observational studies are thus vital for determination of the appropriate use of drugs. In addition, effective and prompt communication of these findings is essential so that the information needed at the medical frontline is supplied to ensure that package inserts, containing basic pharmaceutical data, function as real-time sources of information. To this end, the PMDA framework should be reinforced and detailed post-marketing information gathered for analyses of the pooled data.

NON-CLINICAL SAFETY ASSESSMENT OF METABOLITES

Efforts in Japan

At present, no guidance is available in Japan on the performance of toxicity studies with drug metabolites, so these are handled on a case-by-case basis. The basic viewpoint is that safety verification is required in cases such as the following: (1) those in which metabolites not found in animals occur in humans, (2) those in which the ratios of the metabolites formed in humans and animals differ and metabolites are formed in greater numbers in humans (and where safety cannot be assured on the basis of the metabolite exposure in animal studies), and (3) those in which metabolites considered to have very strong pharmacological or toxicological effects are formed. In cases in which the metabolite of concern is a known substance and its safety profile is clear, or in which the metabolite concerned is deemed to be pharmacologically inactive, or in which the amount formed within the body or exposure to it is believed to be very low, verification of the safety of metabolites may not be required.

The data for new drugs approved in recent years (2000 ~ 2006) were investigated as to the safety assessment of metabolites, study details and rationales, and trends toward assessment of the safety of metabolites. It was found that metabolite safety testing had been conducted on 48 (39%) of the 123 drugs containing new active ingredients (excluding recombinant drugs and biological preparations etc.). Of the 48 products that were investigated for metabolite safety, 44 single-dosing studies (92%) were performed, 7 repeat-dosing studies (15%), and 15 genotoxicity studies (31%) (with some duplication). The most common reason for performing the studies which was given for 44 studies was "It is the major metabolite". Other reasons given were: "It is a unique human metabolite"; "Since virtually no metabolites were formed in rats"; "Since the metabolite was suspected to exhibit genotoxicity"; and "Reason unknown". In the studies investigated, no products were found to have toxicity associated with metabolites that greatly exceeded that of the parent compound. These findings provide further evidence that the number of products for which metabolites exhibit significantly higher toxicity than the parent compound is very small. Nevertheless, these data were obtained for approved drugs, and thus potentially hazardous products suspected of a high degree of metabolite toxicity may have been among the dropouts in the course of drug discovery and development. Future considerations of metabolite toxicity may have been necessary if these dropouts had continued in development.

Safety assessment of metabolites

The process of drug metabolism, excluding prodrugs, generally involves detoxification leading to reduction of pharmacological activity and elimination of the compound. Therefore, cases in which toxicities of a metabolite are completely different from those of the parent compound are believed to be rare. Furthermore, as shown by the examination of new drug approval data referred to above, very few products were noted to have unique human metabolites or to form metabolites that had significantly higher toxicity than the parent compound, thus it is believed that these cases are very rare. However, in some cases in clinical practice there have been concerns regarding manifestations of toxicity ascribable to metabolites, so safety assessment of metabolites found in humans appears to be necessary in these cases. In the FDA draft guidance, the rationale given for the 10-percent criterion in requiring safety assessment of metabolites is based on the amounts of metabolites formed with halothane, felbamate, cyclophosphamide, and acetaminophen; although other examples were provided in which signs and symptoms of toxicity were found with amounts of metabolites less than 10 percent. Accordingly, metabolites found only in humans should basically be subject to safety assessment using some type of method. Furthermore, since in some cases metabolites present in amounts less than 10 percent have been involved in manifestations of toxicity, investigations of the toxicity of these metabolites are called for. In this connection, we have investigated below cases in which toxicity studies of metabolites would be required.

1) Definition and method of assessment of metabolites

Phase I metabolites are formed through reactions

of oxidation, reduction, and hydrolysis, and Phase II metabolites through conjugation reactions. With the exclusion of *N*-acetylization, metabolism involves reactions leading to more polar compounds. Of the conjugate metabolites formed from Phase II reactions, glucuronide conjugates are generally less active than the parent compound. This is thought to be due to the significant molecular modification and increased polarization of aglycones, the decreased affinity of conjugates for receptors and enzymes due to steric hindrance, their reduced membrane permeability (tissue transferability), and the increased ease of their excretion (reduced exposure). It is therefore generally assumed that metabolite toxicity studies using conjugate metabolites are not required. However, cases exist in which there have been concerns over the formation of reactive metabolites in the form of acyl glucuronides or glutathione conjugates, or in which compounds such as irinotecan glucuronide conjugate are deconjugated by enteric bacteria in the intestinal tract after biliary excretion (Takasuna *et al.*, 1996). Thus, adverse reactions need to be fully identified in clinical studies.

Pharmacologically active metabolites are defined as circulating metabolites that have some pharmacological activity. In cases in which metabolites have pharmacological activity similar to or less than the parent compound, it is assumed that safety data have been obtained in the toxicity studies with the parent compound. However, in cases in which the pharmacological activity of the metabolite is greater than that of the parent compound, or in which the metabolite has pharmacological effects different from those of the parent compound, considerations in deciding the necessity for metabolite toxicity studies should include the exposure in humans and any differences between humans and animals in plasma concentration of the pharmacologically active metabolites. In addition, when adverse reactions are noted in clinical practice that cannot be predicted from the parent compound, or when there are strong concerns over the possibility of a relationship between such reactions and a metabolite, the assessment of metabolite toxicity may be needed. Valuable information is sometimes obtained from *in vitro* studies on receptors, enzyme inhibition, or non-clinical *in vivo* metabolite toxicity studies.

Reactive metabolites are intermediate metabolites formed in the process of metabolism that undergo covalent reactions with other physiological substances (proteins etc.). In the safety assessment of reactive metabolites, the investigations undertaken and inferences made from the study results should be based on the test systems in which the reactions occur *in vivo*. It may thus be of little use to determine toxicity through administration to laboratory animals of the final metabolite conjugate instead of the reactive metabolic intermediate. In addition, given the difficulty of chemically synthesizing reactive metabolites formed as metabolic intermediates, *in vitro* toxicity studies incorporating metabolic systems (S9 [9,000 × g supernatant fraction], microsomes, hepatocytes, liver slices) should be considered and studies conducted to appropriately evaluate potential toxicities of the metabolites. However, estimation of the relationships that may exist between clinical adverse events and reactive metabolites is by no means easy; thus, caution should be used when interpreting and extrapolating the *in vitro* findings to clinical findings. Further, consideration of the pathway by which metabolism and elimination occurs is also essential in assessing metabolites. For example, since metabolites excreted in urine are prone to being excreted with polar radicals attached, it is preferable to examine metabolite structure rather than amount of excretion.

Given the differences of opinion found in the public comments studied by the JPMA concerning the 10-percent criterion for safety testing of metabolites described in the FDA draft guidance, and the discrepancy in the value of the metabolite formation ratio established by PhRMA that would require metabolite safety assessment (FDA CDER, Guidance for Industry, Safety Testing of Drug Metabolites (Draft). 2005), it would appear that no clear and agreeable general criterion has been established. Further, studies on humans using radiolabeled forms (RI) are required to determine the metabolite formation, although there are no requirements for these studies in Japan, human RI data have been submitted for many of the products applied for recently. The studies provide important metabolite information used in determining whether or not to conduct animal studies on metabolites or to examine the toxicity of processes of metabolism as they relate to the causes of toxicity obtained from the overall assessment of adverse reactions, signs and symptoms of toxicity, and other manifestations in clinical studies.

2) Safety assessment in laboratory animals

In cases in which metabolite exposure has already been demonstrated in the toxicity studies with the parent compound, follow-up metabolite toxicity studies are usually considered unnecessary. However, in cases in which plasma metabolite exposure in animals is far less than in humans, the metabolite safety

cannot be assured from the results of studies using the dosages of parent included in the animal studies. Therefore, verification of safety is required when there are concerns over the toxic effects of metabolites. In some cases, metabolite exposure may be able to be increased by changing the dosage of the parent compound administered to animals, the number of doses, the rate of administration, or the route of administration.

Even when the safety of unique human metabolites cannot be verified in animal toxicity studies of the parent compound, the safety will at least be demonstrated in clinical studies for the duration and exposure used in the clinical study in which the metabolites were detected. When this occurs, the safety assessment of unique human metabolites will follow a different strategy from that of the parent compound. The chemical structure of unique human metabolites should be identified and the plasma concentration in humans determined. *In silico* determination of toxicity based on structure-activity correlations may also be useful at this stage. In addition, data on adverse reaction reports from clinical studies is essential, and these may lead to further metabolite safety studies.

While the findings of clinical studies are useful, they are limited to results from non-invasive examinations, whereas in animal studies a final necropsy can be performed, allowing tissue damage to be pathologically identified. Such animal studies are therefore essential, in that data unable to be obtained in humans can be obtained in the animals. When adverse events in humans are noted, it is essential in some cases to attempt to correlate these with the results of non-clinical studies. In addition, in many cases it is useful if scientifically valid metabolite toxicity studies can be conducted. When unique human metabolites exist, it may also be useful in some cases to compare the toxicity of the parent compound to that of the metabolite in single-dose, two-week or other toxicity studies.

3) Issues related to metabolites in clinical practice

Drugs given in low doses clinically are generally assumed to yield low metabolite exposure and correspondingly few concerns regarding manifestations of toxicity. Conversely, drugs given in high doses clinically require attention to the possibility of adverse reactions ascribable to metabolites.

In addition, humans are genetically diverse and exhibit individual variations in levels of expression of metabolic enzymes or in metabolic activity. Furthermore, various types of disorders (hepatic, renal, cardiac etc.) may lead to significant variability in pharmacokinetics from that of normal subjects resulting in variations in the parent compound or metabolite plasma concentrations. In addition, drug interactions may occur resulting in variations in the extent of metabolite exposure depending on other factors such as concomitant medications, diet, or lifestyle-related diseases.

In cases in which the metabolite of concern is formed by metabolic enzymes known to exhibit ethnic variations, differences in exposure to the parent compound as well as metabolites between Japanese and other subjects may exist. In such cases, caution is needed in extrapolating data from clinical studies conducted elsewhere to Japanese subjects.

Timing of non-clinical studies on metabolites

The prompt supply of high-quality drugs to the medical frontline is of crucial importance to patients. Non-clinical studies are of vital importance in ensuring the safe administration of drugs in clinical studies. To this end, the timing of non-clinical studies is essential in the process of drug development. Ideally, during the exploratory stage prior to the first administration to humans (First Human Dose and Phase I clinical studies), screening for metabolic stability and for pharmacologically active metabolites should take place, so that compounds selected will give little cause for concern regarding metabolite toxicity. In addition, *in vitro* metabolic studies on S9, microsomes, hepatocytes, liver slices, and other samples obtained from the liver of humans, mice, rats, dogs, monkeys, and other species will allow metabolic profiles to be identified, metabolic species differences to be investigated, and further metabolite characterization using LC-MS/MS, LC-NMR, and other techniques. Based on these results, the decision on which animal species to use in non-clinical safety studies can be determined by comparing the degree of similarity in human and animal metabolism (metabolite formation, metabolic rate). In addition, to avoid toxicity, it may also be useful to screen for reactive metabolites referred to above and to select compounds with minimal risk of formation of such metabolites.

Human metabolites formed *in vivo* can only be confirmed after administration of the drug to humans. In Phase I clinical studies, human samples (plasma, urine, feces) can be evaluated for the presence of metabolites and the metabolite's potential structure identified. Identifying the major human metabolites and assessing for the presence of any unique human

metabolites is of paramount importance. Once the potential metabolites are identified, then an estimation of their toxicity can be made by comparing the chemical structure of the metabolite with that of the parent compound. When appropriate, estimation of the changes in plasma concentrations of metabolites will also be essential. However, detection of changes in the metabolite concentration over time may require longer-term studies. A fully characterized synthesized reference standard of the identified metabolite will need to be provided, and further identification studies of metabolites separate from the clinical study may also need to be considered.

Studies using RI are useful for investigating the extent of metabolite exposure in humans. Microdosing (MD) studies using very low levels of RI may provide the ratio of the parent compound or metabolite to the total exposure in humans at an early date since the MD studies can often be conducted at an early stage of development. Use of MD studies facilitates the early detection of unique human metabolites or the need for further analyses of the metabolite concentrations, and results from these studies in conjunction with the TK analysis can provide useful information on the assessment of metabolite safety.

In cases in which there are strong concerns over the involvement of metabolites in toxicity in humans, it is necessary to carefully examine the findings available on metabolite safety prior to commencement of large-scale clinical studies (Phase III). The FDA guidance recommends that findings on the safety assessment of metabolites be submitted before commencing Phase III clinical studies, especially when unique human metabolites are found that present toxicological problems. The safety of these unique human metabolites in animals should be verified prior to Phase II clinical studies, and if possible, performance of human RI studies should also be considered early in development.

Prior to filing a new drug application (NDA), the metabolic enzymes and metabolic pathways of major human metabolites of a drug should be determined. The metabolite structure should be identified and the exposure in humans and animals should be determined using blood samples. The pharmacological activity of the metabolite should be assessed as well. Additionally, an understanding of whether or not the metabolites are unique to humans is essential and if so, then additional studies may be warranted.

After launch of a new drug, post-marketing surveillance studies should be carefully performed and are essential to verify drug efficacy and safety in daily medical care as they provide a means to collect information on the appropriate use of drugs unable to be obtained in clinical trials prior to marketing (Table 4). In particular, serious adverse events reported after a new drug launches may include those which non-clinical or clinical study results did not predict. In this connection, for adverse events found in clinical practice, it may be necessary to carefully examine unpublished results and results not available at the time of the application for causal links.

Table 4. Findings of post-marketing surveillance.

Safety and usefulness on the market
Long-term toxicity that affects individuals up to the next generation
Extremely rare, serious, or unpredictable adverse effects
Frequency of adverse effects (time course)
Identification of new types of efficacy
Off-label use
Overdose
Medical economics of drug treatment

Issues concerning methods of metabolite assessment

Studies will sometimes have to be conducted to assess metabolite safety. However, in some cases adequate exposure cannot be obtained even with administration of the metabolite to animals using the route of administration of the parent compound, or only transient exposure can be obtained with intravenous treatment. Exposure to metabolites may require significant modification through such methods as changing the route of administration, the dosage, or the number of administrations, etc. However, notwithstanding the apparent degree of exposure in the systemic circulation, changes in tissue transferability associated with such factors as plasma protein binding, membrane permeability, or affinity for transporters may reduce exposure in the target tissue, resulting in an underestimation of the toxicity. Moreover, the toxicity of reactive metabolites that give cause for concern may not be able to be studied by administering the conjugate metabolite to animals. There are also technical problems, in that chemical synthesis of reactive metabolites and glucuronide conjugates, etc. is often difficult and synthesized reference standards of metabolites are sometimes impossible to prepare. In view of the practical problems such as these, studies of the feasibility of the safety assessment of metabolites are required. In some cases, the profile of toxicity of a metabolite will have to

be determined from numerous factors, including *in silico* predictions from chemical structure databases or *in vitro* studies incorporating human metabolic systems. The safety assessment of metabolites requires consideration of complex factors, and even when safety testing of metabolites is deemed unnecessary, a full explanation of the rationale will need to be provided.

FUTURE DEVELOPMENT

In the interest of delivering high-quality drugs to patients as quickly as possible, safety assessment of metabolites was discussed at the kick-off meeting of the Drug Evaluation Forum, with reference to the views of clinicians and other academic representatives, in order to provide useful information for developers and reviewers. In the course of drug development, safety in humans is predicted through means such as metabolite profiling and pharmacological activity screening, clinical pharmacokinetic studies, human RI studies, in addition to the safety studies using metabolite reference standards which are conducted when deemed necessary. Although at present there are no guidelines for metabolite safety testing in Japan, 40 percent of the compounds in licensed products have undergone some form of safety testing of metabolites, and few metabolites unique to humans or with significant toxicity appear to have been found. However, these findings are based only on products approved as new drugs and do not include cases where development of a compound was discontinued. Indeed, it is likely that a large amount of valuable information on metabolites exists in the latter cases. Accordingly, with the cooperation of pharmaceutical companies, it is essential to collect and pool data on the toxicity of metabolites, especially negative data. Establishment of a standard database could be part of the consideration when drafting of guidelines, etc., and preferably a database that will facilitate worthwhile safety assessment. In Japan, as well, pharmaceutical companies would create databases, including negative data, based on consensus regarding their rôle in contributing to the needs of society through the prompt supply of safe and effective drugs to patients. In addition, on the regulatory sciences front, post-marketing surveillance enables prompt communication of findings to prescribers and patients, and it is also advisable to collect data on metabolites during clinical trials.

It is of vital importance to analyze metabolites using human samples obtained in clinical studies and to determine whether unique human metabolites are major metabolites. However, since these tasks may also constitute a rate-limiting factor in drug development, in some cases drug risk-benefit considerations may require selection of the optimal strategy through use of PMDA consultations or other services. In general, even when unique human metabolites or major metabolites are found in clinical studies, these studies are often single-dose, with smaller dosages than in non-clinical safety studies, or the safety investigations are based on short-term clinical studies with few reported adverse reactions. It therefore cannot always be assumed that the safety of metabolites in humans has been verified. Moreover, after approval, a marketed drug will be used by large numbers of patients and in some cases will be administered long-term. The principal importance of safety studies is identification of the toxicity profile and estimation of safety in humans after administration of high dosages for adequate periods of time. Further, since in the course of drug development the human metabolite profile is often identified around the conclusion of clinical Phase I studies, it is conceivable that development may be slowed when results on metabolites are obtained. On the other hand, MD studies may be helpful in metabolite profiling, and may accelerate assessment of human metabolites. In addition, reactive metabolites, even if a problem in clinical practice, are often unverifiable in ordinary toxicity studies. Provisions should be made for screening systems using human samples that may better evaluate the potential for reactive metabolites and allow a comparison of the findings on reactivity in humans to the findings in animals.

Clinically, a seemingly small finding with use of drugs in humans may have a large impact, so it is essential not to overlook problems that arise at the medical frontline. Future challenges will include how to reduce clinical risks based on considerations of whether clinical problems with less frequency can be detected in the non-clinical studies, or how clinical information should be used to impact basic research. Although compounds supplied to the medical frontline as drugs have been assessed for safety through toxicity studies, post-marketing clinical problems not observed during drug development may occur and may differ considerably from those found during daily usage by large populations. Therefore, feedback to drug researchers of findings from the medical frontline could, for example, be important information useful for improving drug development. Nevertheless, the incidence of actual problems in patients is very low, and these may be problems that cannot be detected by

the non-clinical studies. Accordingly, in the event of adverse effects, it is essential to act as quickly as possible to find ways to understand the mechanism behind the event.

In these terms, the drafting of strict guidelines on the safety assessment of metabolites presents considerable difficulties. It may be preferable to prepare flexible guidelines to speed the development of useful drugs. However, while such guidelines should be scientifically valid, it may be difficult to establish fixed criteria for assessment of the safety of metabolites. The safety assessment of metabolites cannot readily be based on a single theoretical framework, and basically a case-by-case approach is called for. However, some common understanding should be shared. To this end, it is useful to first prepare an outline concept paper. A collective viewpoint is also relevant to any discussion on the need for guidance, etc. A degree of flexibility may also be helpful for drug development and, in turn, society at large. The safety of metabolites should be assessed using scientifically sound methods and take into account signs, symptoms and adverse reactions that may be unpredictable in clinical studies on the parent compound, but may be related to the metabolites. In addition, elimination of scientifically meaningless studies for the safety assessment of metabolites is essential for the prompt supply of high-quality drugs to the medical frontline.

ACKNOWLEDGMENT

Our sincere thanks to those who helped to promote the kick-off meeting of the Drug Evaluation Forum (Masataka Mochizuki, President of Kyoritsu College of Pharmacy; Satoshi Toyoshima, Director of the Center for Product Evaluation, Pharmaceuticals, and the Medical Devices Agency; Kazuhiko Nakashima, Chairperson of the Drug Evaluation Committee, JPMA; Toshihiko Kobayashi, Japan Technical Representative of the Pharmaceutical Research and Manufacturers of America; and Hajime Iwasaki, European Federation of Pharmaceutical Industries and Associations Japan), Taskforce 11 of the JPMA Non-clinical Evaluation Subcommittee of Drug Evaluation Committee, and those concerned at EFPIA-J and PhRMA-J. Our sincere thanks also to Kazuko Soshiki and all members of the Kyoritsu College of Pharmacy, the cosponsor, for their tremendous efforts in running the Forum. In addition, our sincere thanks to all those who gave pertinent advice and polite comments on numerous occasions in the preparation of this summary.

REFERENCES

Baillie, T.A., Cayen, M.N., Fouda, H., Gerson, R.J., Green, J.D., Grossman, S.J., Klunk, L.J., LeBlanc, B., Perkins, D.G. and Shipley, L.A. (2002): Drug metabolites in safety testing. Toxicol. Appl. Pharmacol., **182**, 188-196.

Baillie, T.A., Cayen, M.N., Fouda, H., Gerson, R.J., Green, J.D., Grossman, S.J., Klunk, L.J., LeBlanc, B., Perkins, D.G. and Shipley, L.A. (2003): Reply. Toxicol. Appl. Pharmacol., **190**, 93-94.

CPMP/EWP/560/95, 17, December (1997): The note of guidance on investigation of drug interactions, Committee for Proprietary Medicinal Products.

Davis-Bruno, K. L. and Atrakchi, A. (2006): A regulatory perspective on issues and approaches in characterizing human metabolites. Chem. Res. Toxicol., **19**, 1561-1563.

FDA CDER Guidance for Industry, Published in Federal Register, March (2003): Guidance for Industry; Bioavailability and Bioequivalence Studies for Orally Administered Drug Products - General Considerations.

FDA CDER Guidance for Industry, Published in Federal Register, June (2005): Guidance for Industry, Safety Testing of Drug Metabolites (Draft).

FDA CDER Guidance for Industry, Published in Federal Register, September (2006): Guidance for Industry; Drug Interaction Studies – Study Design, Data Analysis, and Implications for Dosing and Labeling (Draft).

Guengerich, F.P. (2006): Safety Assessment of Stable Drug Metabolites. Chem. Res. Toxicol., **19**, 1559-1560.

Hastings, K.L., El-Hage, J., Jacobs, A., Leighton, J., Morse, D. and Osterberg, R.E. (2003): Letter to the Editor. Toxicol. Appl. Pharmacol., **190**, 91-92.

Humphreys, W.G. and Unger, S.E. (2006): Safety assessment of drug metabolites: Characterization of chemically stable metabolites. Chem. Res. Toxicol., **19**, 1564-1569.

Inoue, T., Harada, Y., Furuta, S., Kawamura, Y., Kurihara, A., Kurokawa, M., Nakazawa, T. and Sagami, F. (2005): The industry's concerns on the inquiries derived from the review of non-clinical studies - Analysis of questionnaire survey data by JPMA, Pharm. Regul. Sci, **36**, 388-397.

Iyakushin no. 496, Jun. 26th, 1998: Guidance for non-clinical Pharmacokinetic studies.

Lazarou, J., Pomeranz, B.H. and Corey, P.N. (1998): Incidence of adverse drug reactions in hospitalized patients: a meta-analysis of prospective studies. JAMA., **15**, 279, 1200-1205.

Ohno, Y. (1992): Toxicity testing: Regulatory Perspectives. Proceedings of the First International Conference on Harmonisation. 1991 Nov.; Brussels, Belgium. Ed D'Arcy PFD and Harron DWG. The Queen's University of Belfast. 186-188.

Prueksaritanont, T., Lin, J.H. and Baillie, T.A. (2006): Complicating factors in safety testing of drug metabolites: Kinetic differences between generated and preformed metabolites. Toxicol Appl Pharmacol., **217**, 143-152.

Smith, D.A. and Obach, R.S. (2005): Seeing through the mist: Abundance versus percentage. Commentary on metabolites in safety testing. Drug Metab Dispos., **33**, 1409-1417.

Smith, D.A. and Obach, R.S. (2006): Metabolites and safety: What are the concerns, and how should we address them? Chem. Res. Toxicol., Dec., **19**, 1570-1579.

Takasuna, K., Hagiwara, T., Hirohashi, M., Kato, M., Nomura, M., Nagai, E., Yokoi, T. and Kamataki, T. (1996): Involvement of β-glucuronidase in intestinal microflora in the intestinal toxicity of the antitumor camptothecin derivative irinotecan hydrochloride (CPT-11) in rats. Cancer Res., **56**, 3752-3757.

The Japanese Society for the Study of Xenobiotics (JSSX, November 29th - December 1st, 2006, Tokyo): 21st Annual Meeting Abstracts 171-177.

The Pharmaceutical Society of Japan (Jan. 13th, 2007, Tokyo): Kick-Off Meeting of Drug Evaluation Forum - Toward an Innovative Medicine: From Non-clinical to Clinical. Abstracts.

Toxicology Q&A (2003): (Question 16 : Toxicological evaluation of unique human metabolites), Pharm. Regul. Sci, **34**, 794-795.

Yakushin no. 526, March 28th, 1975: Points to Consider for Approval Application Data for New Manufacture or Import Drugs.

Yamada, H., Yamaguchi, J., Iida, I. and Okuyama, S. (2006): Idiosyncratic drug toxicity, Folia Pharmacol. Jpn., **127**, 473-480.

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

A Reliable and Sensitive Immunoassay for the Determination of Crustacean Protein in Processed Foods

KOSUKE SEIKI,[†] HIROSHI ODA,[†] HISASHI YOSHIOKA,[†] SHINOBU SAKAI,[‡] ATSUO URISU,[§] HIROSHI AKIYAMA,*[,‡] AND YASUO OHNO[‡]

Central Research Institute, Maruha Nichiro Holdings, Inc., 16-2, Wadai, Tsukuba, Ibaraki 300-4295, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, and The Second Teaching Hospital, Fujita Health University, 3-6-10 Otobashi, Nakagawa-ku, Nagoya, 454-8509, Japan

Among food allergens, crustacea such as shrimps, crabs, and lobsters are a frequent cause of adverse food reactions in allergic patients. The major allergen has been identified as a muscular protein, tropomyosin. A novel sandwich enzyme-linked immunosorbent assay (ELISA) for the detection and quantification of crustacean protein in processed foods was developed using the sample dilution buffer that is added to porcine tropomyosin. The sandwich ELISA method was highly specific for the Decapoda group, apart from minor cross-reactivities to other crustacea and mollusks. The recovery ranged from 85 to 141%, while the intra- and interassay coefficients of variation were less than 2.8 and 8.4%, respectively.

KEYWORDS: Crustacea; food allergy; enzyme immunoassay; ELISA; tropomyosin

INTRODUCTION

In industrialized countries, food allergies have represented an important health problem in recent years, and it is estimated that approximately 8% of children and 2% of adults have some type of food allergy (*1*, *2*). Burks et al. (*3*) estimated that approximately 120 deaths related to food allergies occur yearly in the United States. In Japan, the number of patients with food allergies, especially among young children, is increasing.

To prevent possible life-threatening reactions, the only effective treatment is to strictly avoid the consumption of these allergenic foods. However, various studies have shown that severe allergenic reactions can be caused by the accidental intake of food products containing allergenic materials (*4*, *5*). Therefore, sufficient information regarding potentially allergenic ingredients in food products is necessary. The issue of a labeling system for allergenic ingredients in food products has been discussed by international organizations, such as the Codex Alimentarius Commission of the World Health Organization (WHO) and the Food Agriculture Organization (Codex 1998). In 1999, the Joint FAO/WHO Codex Alimentary Commission Session agreed to label eight kinds of foods that contain ingredients known to be allergens, including soybeans (FAO 1995, *6*). In Japan, the Ministry of Health, Labor, and Welfare (MLHW) has enforced a labeling system for allergenic food material since April 2002 to provide information about these foods to the allergic consumer. In this system, labeling for five food products, including eggs, milk, wheat, buckwheat, and peanuts, is mandatory and is recommended for 20 other food materials, such as soybeans and shrimp. In Japan, it became clear, based on epidemiological investigations, that the number of patients with a crustacean allergy such as to shrimp or crab has increased (*7*, *8*).

In recommendations regarding labeling, Crustacea labeling would be particularly important because of the almost unlimited uses of Crustacea and because the number of patients with an allergy to Crustacea has been increasing, although the crustacean allergy is still less prevalent than the peanut allergy in the food-allergic population (*9*, *10*). Crustacean allergic reactions may occur due to trace amounts of the crustacean protein, and anaphylaxis to Crustacea has been reported (*11*, *12*).

In the present study, we developed a reliable sandwich enzyme-linked immunosorbent assay (ELISA) method with a high sensitivity for Crustacea. We showed that this detection method could be applicable to food-processing products and that the trace amount of Crustacea contained in commercial food products can be detected using the proposed sandwich ELISA method.

MATERIALS AND METHODS

Food Samples. The black tiger prawn (*Penaeus monodon*) was purchased from Intergrated Aquaculture Specialist, Inc. (Cebu, Philippines). The common Crustacea and mollusks, namely, northern shrimp (*Pandalus borealis*), Japanese spiny lobster (*Panulirus japonicus*),

* To whom correspondence should be addressed. Tel: +81-03-3700-9397. Fax: +81-03-3707-6950. E-mail: akiyama@nihs.go.jp.
[†] Maruha Nichiro Holdings, Inc.
[‡] National Institute of Health Sciences.
[§] Fujita Health University.

10.1021/jf0715471 CCC: $37.00
Published on Web 10/12/2007

Caribbean spiny lobster (*Panulirus argus*), red king crab (*Paralithodes camtschatica*), swimming crab (*Portunus trituberculatus*), Japanese mantis shrimp (*Squilla oratoria*), euphausia (*Euphausia similis*), opossum shrimp (*Neomysis japonica*), acorn barnacle (*Balanus rostratus*), goose barnacle (*Pollicipes mitella*), common octopus (*Octopus vulgare*), giant octopus (*Paroctopus dofleini*), ocellated octopus (*Octopus ocellatus*), Japanese common squid (*Todardes pacificus*), spear squid (*Loligo kobiensis*), cuttlefish (*Sepia subaculeata*), common scallop (*Patinopecten yessoensis*), Japanese oyster (*Crassostrea gigas*), bloody clam (*Scapharca broughtonii*), blue mussel (*Mytilus edulis*), short-neck clam (*Tapes japonica*), common freshwater clam (*Corbicula leane*), Japanese hard clam (*Meretix lusoria*), Sakhalin surf clam (*Spisula sachalinensis*), horned turban (*Turbo cornutus*), Japanese abalone (*Haliotis discus*), and whelk (*Babylonia japonica*), and other ingredients and commercial processed foods were purchased at local stores in Japan.

Preparation of Model Processed Foods. The model processed foods were prepared according to a previously reported cooking procedure (*13*). Specifically, the freeze-dried black tiger prawn muscle powder was mixed with raw foods and then cooked to prepare the processed food items containing the shrimp protein at 10 μg/g as the final concentration. The following were prepared as the model processed foods.

Fish meat sausages were made of minced fish flesh (yellow goatfish, atka mackerel, and walleye pollack), lard, sugar, salt, ice water, and the spiking powder. Lard, salt, sugar, ice water, and the spiking powder were added to the minced fish flesh and thoroughly mixed. The mixture was ground using a small cutter, and the kneaded mixture was manually placed into the fish sausage casings. These were then heated at 121 °C for 15 min, cooled in flowing water for 5 min, and then placed in a refrigerator at 5 °C overnight.

The freeze-dried egg soup was made of egg, potato starch, milk sugar (lactose), salt, and the spiking powder. Eggs, potato starch, milk sugar, salt, and the spiking powder were thoroughly mixed. The mixture was dissolved in a plastic tray, frozen in a deep freezer at −80 °C, and then lyophilized for 20 h.

The chicken meatball was made of white meat of chicken, lard, potato starch, sugar, and the spiking powder. Lard, potato starch, sugar, and the spiking powder were added to ground white meat of chicken and thoroughly mixed. The mixture was ground using a small cutter, and the kneaded mixture was manually placed into casings. These were then preserved in a deep freezer at −20 °C.

Preparation of Black Tiger Prawn Protein Standards. A 0.1 g sample of the freeze-dried black tiger muscle powder was added to 20 mL of phosphate-buffered saline [10 mM Na-phosphate, 154 mM NaCl (pH 7.4)] containing 5 g/L sodium dodecyl sulfate (SDS), 20 mL/L β-mercaptoethanol, 10 μL/mL protease inhibitor cocktail, and 10 μL/mL 0.5 M ethylenediaminetetraacetic acid (Halt protease inhibitor cocktail kit; Pierce, Rockford, IL). The mixture was then shaken for 15 h at room temperature for extraction. After the extraction, the sample was centrifuged at 10000*g* for 30 min and the supernatant was filtered through a 0.8 μm microfilter paper (DISMIC-25cs; Advantec, Tokyo, Japan) to obtain the extract. The extract was then heated at 100 °C for 10 min. The obtained extracts were analyzed using a 2D Quant Protein Assay Kit (GE Healthcare UK Ltd. NA, England).

Purification of Black Tiger Prawn Tropomyosin (BTTM), Red King Crab, Swimming Crab, Japanese Oyster, Common Scallop, Japanese Common Squid, and Porcine Tropomyosin (PTM). The purification of the BTTM was carried out according to the methods reported by Nagpal et al. (*14*), Ishikawa et al. (*15*), and Miegel et al. (*16*), respectively. The black tiger prawn muscles (100 g) were homogenized with 200 mL of a solution containing 20 mM KCl, 1 mM $KHCO_3$, 0.1 mM $CaCl_2$, and 0.1 mM dithiothreitol (DTT). After centrifugation (3000*g* for 5 min at 4 °C), 200 mL of acetone was added to the precipitant. The suspension was filtered through cheesecloth, and the residue was then washed three times with 200 mL of acetone. Finally, the residue was allowed to dry at room temperature for 2–3 h. The dried powder was extracted overnight at room temperature with 200 mL of 25 mM Tris-HCl buffer (pH 8.0) containing 1 M KCl, 0.1 mM $CaCl_2$, and 1 mM DTT. After filtration through cheesecloth, the residue was once more extracted with 200 mL of 1 M KCl. The extracts were combined and cooled to 4 °C. Ammonium sulfate was added to produce an approximate 30% saturation. After 2 h, the solution was centrifuged (18000*g* for 60 min at 4 °C) and ammonium sulfate was then added to the supernatant (60% saturation). After 2 h, the solution was centrifuged and the precipitant was dissolved in 20 mL of 5 mM Tris-HCl (pH 7.5) containing 0.1 mM $CaCl_2$, and 0.1 mM DTT and was dialyzed overnight against 6 L of the same solution. The pH was then adjusted to 4.6 by the addition of HCl, and the tropomyosin precipitate was removed by centrifugation. The precipitate was dissolved in 25 mM Tris-HCl (pH 8.0) containing 1 M KCl, 0.1 mM $CaCl_2$, and 0.1 mM DTT and then chromatographed on a HiLoad Superdex 200 pg column (Φ26 mm × 600 mm; GE Healthcare UK Ltd.) equilibrated with the same buffer. Fractions of 5 mL were collected at a flow rate of 2.5 mL/min. The SDS-polyacrylamide gel electrophoresis analyses for all fractions were performed, and the fractions with the band corresponding to 37 kDa were combined (*17*). The combined fraction was then diluted with an equal volume of 0.2% trifluoroacetic acid and applied to reverse-phase high-performance liquid chromatography on a Wakosil-II 5C18 AR prep column (Φ10 mm × 250 mm; Wako Chemicals, Japan). The column was eluted at a constant flow rate of 2.5 mL/min by a gradient of acetonitrile in 0.1% trifluoroacetic acid. The tropomyosin-containing fractions were collected and lyophilized. The red king crab, swimming crab, Japanese oyster, common scallop, and Japanese common squid tropomyosins were obtained according to the purification procedure of BTTM. Tropomyosin derived from the porcine skeletal muscle (PTM) was obtained using the purification procedure of Greaser et al. (*18*) and Bailey et al. (*19*).

Production of Monoclonal Antibodies and Rabbit Polyclonal Antibodies to BTTM. The anti-BTTM monoclonal antibodies were generated at Nippon Biotest Laboratories, Inc. (Tokyo, Japan). For the production of the monoclonal antibodies against BTTM, female BALB/c mice were immunized with the purified BTTM. Fusion of the spleen cells was performed according to the method of Kohler and Milstein (*20*). The cell culture supernatants were screened for specific anti-BTTM antibodies by a direct ELISA with purified BTTM on a solid phase. The positive hybridomas were cloned and subcloned by limiting dilution. The positive hybridoma cells were intraperitoneally administered into BALB/c mice to induce the ascite tumors. The antibody was purified from the ascite fluid using a HyperD Protein A column (Bio Sepra Inc., Marlborough, MA). The specificity of the monoclonal antibodies was demonstrated by a direct ELISA method with purified black tiger prawn, red king crab, Japanese oyster, common scallop, and Japanese common squid tropomyosins. The polyclonal antibodies were generated at Medical and Biological Laboratories Co., Ltd. (Nagoya, Japan). The rabbit antiserum against BTTM was produced by immunization of New Zealand rabbits with purified BTTM in Freund's adjuvant. Injections were repeated six times at appropriate intervals (7 days). Whole blood was collected, and the serum was separated. The polyclonal antibodies were purified from the serum using a HiTrap Protein A HP column (GE Healthcare UK, Ltd.). The polyclonal antibodies were immunoabsorbed against Japanese common squid purified tropomyosin. The immunoabsorption was performed using the Japanese common squid tropomyosin-coupled column to removed further antibodies to molluskan protein. The specificity of the absorbed polyclonal antibodies was demonstrated by direct ELISA using the various purified tropomyosins.

Preparation of Sample Solution. The samples were treated with the Ace AM-4 homogenizer (Nissei, Tokyo, Japan) a few times for 30 s for homogeneity. Nineteen milliliters of 120 mM Tris-HCl (pH 7.4) containing 1 g/L bovine serum albumin (BSA), 0.5 mL/L Tween 20, 5 g/L SDS, and 20 mL/L β-mercaptoethanol (*21*) was added to 1 g of a homogenized sample, which was then shaken for 12 h at room temperature for extraction. After the extraction, the sample was centrifuged at 3000*g* for 20 min, and the supernatant was filtered through 5AB paper (Advantec) to obtain the extract.

Procedure of the Direct ELISA. Polystyrene 96 well microtiter plates (Nalge Nunc international, Rochester, NY) were coated overnight at 4 °C with 100 μL of purified tropomyosin (0.5 μg/mL) in coating buffer (50 mmol/L sodium carbonate, pH 9.6). The plates were then washed three times with Tris-buffered saline (TBS; 20 mmol/L Tris-HCl, pH 7.4, containing 154 mmol/L NaCl). The plates were blocked

for 1.5 h at 25 °C with TBS containing 10 g/L BSA, 30 g/L sucrose, and 0.5 mL/L ProClin 950 (Supelco, Bellefonte, PA). After the plates were washed six times with TBS containing 0.5 mL/L Tween 20 (TBS-T), diluted monoclonal antibodies or polyclonal antibodies were added to the wells and incubated at 25 °C for 1 h. After the wells were washed with TBS-T, 100 μL of horseradish peroxidase-labeled goat antimouse or antirabbit IgG serum was added to each well. After washing, 100 μL of the substrate solution containing 3,3′,5,5′-tetramethylbenzidine (SureBlue TMB Microwell Peroxidase Substrate; KPL, Gaithersburg, MD) was added to each well, and the plate was incubated at 25 °C for 20 min. The reaction was stopped by the addition of 1 mol/L sulfuric acid (100 μL/well). The plate was then read on a SPECTRAmax 250 microplate reader (Molecular Devices Corp., Menlo Park, CA) at the wavelength of 450 nm.

Procedure of the Sandwich ELISA. Polystyrene 96 well microtiter plates (Nalge Nunc international) were coated with 100 μL/well monoclonal antibodies (Mab #32, 10 μg/mL; and Mab #54, 20 μg/mL in 50 mmol/L sodium carbonate, pH 9.6) for 18 h at 4 °C. After they were washed three times with TBS, the plates were blocked for 1.5 h at 25 °C with TBS containing 10 g/L BSA, 30 g/L sucrose, and 0.5 mL/L ProClin 950. After the blocking buffer had been aspirated, the plates were dried in an incubator for 2.5 h at 30 °C, sealed in an aluminum-coated pack with drying agent (I.D. Sheet Desiccant; I.D., Tokyo, Japan), and stored at 4 °C until used. The food sample extracts were diluted 1:20 with the sample dilution buffer [TBS containing 2 g/L BSA, 0.02 g/L PTM, 0.5 mL/L Tween 20, and 0.5 mL/L ProClin 950]. The diluted sample or calibrator (100 μL) was added in triplicate to the coated wells, and the plates were then incubated for 90 min at 25 °C. After the plate had been washed four times with TBS-T, horseradish peroxidase-conjugated absorbed polyclonal antibodies (100 μL) were added to each well, and the plate was then incubated for 90 min at 25 °C. After another four washes with TBS-T, 100 μL of 3,3′,5,5′-tetramethylbenzidine solution (SureBlue Reserve TMB Microwell Peroxidase Substrate, KPL) as a substrate was added to each well, and the plate was incubated at 25 °C for 20 min. The reaction was stopped by the addition of 1 mol/L sulfuric acid (100 μL/well). The plate was then read using a SPECTRAmax 250 microplate reader at a wavelength of 450 nm. Standard curves were obtained by plotting the absorbance vs the logarithm of the analyte concentration.

Eleven crustacean protein extracts and two crustacean purified tropomyosins were tested in the concentration range from 0 ng/mL to 90 μg/mL. The concentration–response curves were obtained by plotting the absorbance vs the logarithm of the analyte concentration, and the curves were fitted to a four-parameter logistic equation, $y = \{(A - D)/[1 + (x/C)^B]\} + D$, where A is the maximum absorbance at infinite concentration, B is the curve slope at the inflection point, C is the concentration of the analyte giving 50% responses (RC_{50}), and D is the minimum absorbance for no analyte. The reactivity values were calculated as follows: reactivity % = [RC_{50} of black tiger prawn protein (or tropomyosin)/RC_{50} of target crustacean protein (or tropomyosin)]. Seventeen molluskan sample extracts were diluted 1: 20 with the sample dilution buffer containing PTM and analyzed using the sandwich ELISA method.

Evaluation of Assay Variation. For determination of the intra-assay precision, the mean coefficients of variation (CVs) were based on 10 replicates. The interassay precision was determined as the mean CVs on the basis of triplicate analyses on 10 different days. The limit of detection (LOD) for the sandwich ELISA was calculated as three times the standard deviation (SD) of the buffer blank mean value after 25 experiments. The limit of quantification (LOQ) was calculated as 10 times the SD of the buffer blank mean values after 25 experiments.

RESULTS

Construction of Sandwich ELISA. To evaluate the characteristics of the absorbed polyclonal antibodies and monoclonal antibodies to the BTTM, we tested the reactivity using a direct ELISA assay. We showed that the absorbed polyclonal antibody could be clearly detected for the crustacean tropomyosin but not for the molluskan tropomyosin. For the preparation of monoclonal antibodies to BTTM, nine monoclonal antibodies were obtained. Of these monoclonal antibodies, Mab #32 and #54 gave a satisfactory specificity and reactivity. In the examination of the different antibody combinations, using Mab #32 and #54 as the capture antibody and the HRP-conjugated absorbed polyclonal antibody as the detected antibody for the sandwich ELISA was found to provide the best results in terms of sensitivity and specificity to determine the total crustacean protein. However, as shown in **Figure 1**, a satisfactory dilution linearity could not be obtained when the dilution tests were performed using the tentatively constructed sandwich ELISA method and the three model processed foods. These results suggest that the food matrix could affect the dilution linearity in the tentatively constructed ELISA method. Therefore, to improve the dilution linearity, we attempted to add the PTM to the sample dilution buffer. As shown in **Figure 2**, the dilution linearity was satisfactorily improved by the addition of the PTM to the sample dilution buffer (r^2 = 0.996–0.999), confirming parallelism between the calibrators and the food samples. We statistically compared the two correlation coefficients of the dilution curves obtained using a sample dilution buffer containing PTM and those obtained without PTM for the assay of the model processed foods. A statistical test between the two correlation coefficients was performed using the Z-transformation test. P values of less than 0.05 were considered statistically significant. In the case of the chicken meatball, there was a statistically significant difference between the two correlation coefficients of the dilution curve (P = 0.036). In the case of

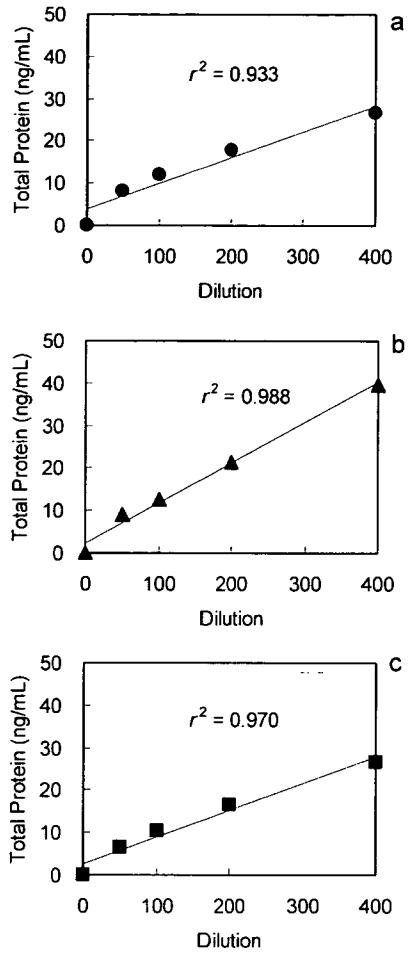

Figure 1. Linearity of dilution curves for model processed foods using the sample dilution buffer without PTM. (**a**) Fish meat sausage (solid circle); (**b**) freeze-dried egg soup (solid triangle); and (**c**) chicken meatball (solid square).

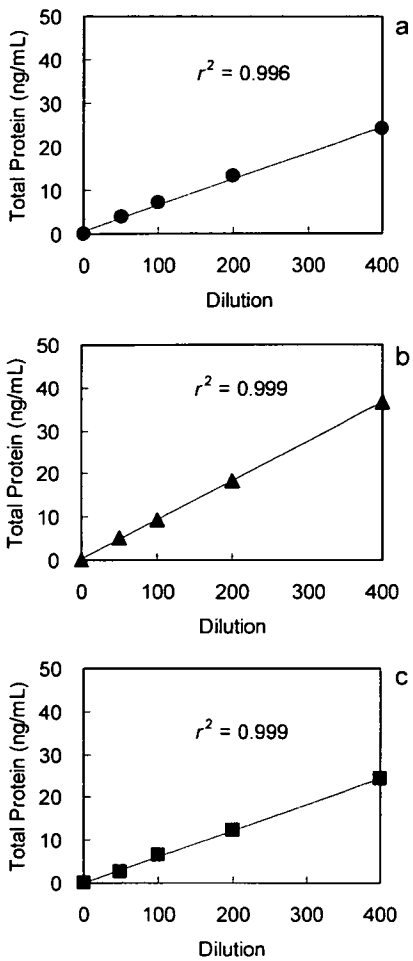

Figure 2. Linearity of dilution curves for model processed foods using the sample dilution buffer with PTM. (**a**) Fish meat sausage (solid circle); (**b**) freeze-dried egg soup (solid triangle); and (**c**) chicken meatball (solid square).

the fish meat sausage, a dilution curve using a sample dilution buffer to which was added PTM tended to show a good linearity when compared with using a tentative sample dilution buffer (P = 0.081). Meanwhile, there was no statistically significant difference in the freeze-dried egg soup (P = 0.127). These results suggested that the addition of PTM to the sample dilution buffer significantly improved the dilution linearity. Consequently, we established the sandwich ELISA method using a sample dilution buffer with PTM to minimize the food matrix effects.

Reactivity and Specificity Test. Various crustacean proteins, molluskan protein samples, and two crustacean purified tropomyosins (black tiger prawn and swimming crab) were examined to test the reactivity and specificity using the sandwich ELISA method. As shown in **Table 1**, the reactivities of the Decapoda group, which includes prawns and lobsters, are greater than 65.8%, and those of the crabs range between 28.5 and 38.5%. In contrast, the reactivities of the other Crustacea, such as the Japanese mantis shrimp, euphausia, and acorn barnacle, are less than 11.3%. The swimming crab purified tropomyosin demonstrated a reactivity of 154% as compared to the reactivity of black tiger purified tropomyosin. When all of the molluskan samples were tested, all of the levels were determined by the sandwich ELISA method to be less than 1.0 mg/kg (**Table 2**). These results suggest that the sandwich ELISA method has a specific reactivity to the Decapoda group, which includes prawns, shrimps, lobsters, and crabs.

Table 1. Reactivity Levels of Various Crustacean Samples in the Sandwich ELISA Method[a]

sample	RC_{50} (ng/mL)	reactivity (%)
Decapoda group		
black tiger prawn	9.5	
northern shrimp	14.4	65.8
Japanese spiny lobster	8.4	114.3
Caribbean spiny lobster	9.0	105.6
red king crab	24.6	38.5
swimming crab	33.4	28.5
other varieties of Crustacea		
Japanese mantis shrimp	124.4	7.6
euphausia	799.3	1.2
opossum shrimp	8060.4	0.1
acorn barnacle	83.8	11.3
goose barnacle	166.7	5.7

[a] RC_{50} is the concentration of analyte giving a 50% OD_{max} response. Reactivity % = (RC_{50} of black tiger prawn protein/RC_{50} of target crustacean protein).

Table 2. Cross-Reactivity of Various Molluskan Samples in the Sandwich ELISA Method

sample	cross-reactivity in ELISA (mg/kg)
Cephalopoda group	
common octopus	<1.0
giant octopus	<1.0
ocellated octopus	<1.0
Japanese common squid	<1.0
spear squid	<1.0
cuttlefish	<1.0
Bivalvia group	
common scallop	<1.0
Japanese oyster	<1.0
bloody clam	<1.0
blue mussel	<1.0
short-neck clam	<1.0
common freshwater clam	<1.0
Japanese hard clam	<1.0
Sakhalin surf clam	<1.0
Gastropoda group	
horned turban	<1.0
Japanese abalone	<1.0
whelk	<1.0

Limit of Detection and Limit of Quantification. The best model that describes the relationship between the absorbance and the antigen concentration is a four-parameter logistic curve (**Figure 3**). The LOD of the ELISA method determined using the standard proteins is 0.71 ng/mL, equivalent to 0.29 mg/kg samples, and LOQ is 2.25 ng/mL, equivalent to a 0.9 mg/kg sample. Consequently, the practical determination range lies between 1.56 and 50 ng/mL. For the final evaluation of the validation data for the sandwich ELISA and its application, the LOQ for routine analysis was set to 1.0 mg/kg sample. This level was considered to give a safety margin to the majority of consumers with an allergy to peanuts (*22*).

Quantification of Crustacean Protein in Model Processed Foods Using the Sandwich ELISA. To test the applicability of the sandwich ELISA in processed foods, the crustacean protein in three model processed food samples was determined using the sandwich ELISA. As shown in **Table 3**, the mean recoveries for all three model processed food samples ranged from 85 to 141%. The precision data from the three model processed foods are shown in **Table 4**. The interassay precision across all days was 5.3, 6.2, and 8.4% CV for the three model processed foods. The intra-assay precision for the three model processed foods was 2.8, 2.3, and 2.8% CV, respectively.

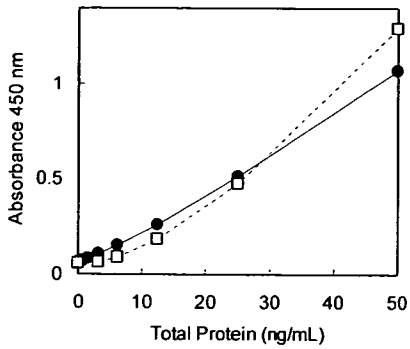

Figure 3. Representative standard curve using the shrimp protein standard in the sandwich ELISA method. The sample diluted buffer with PTM (solid circle); without PTM (open square).

Table 3. Recoveries of Crustacean Protein from Three Model Processed Foods

sample	concentration (mg/kg)	recovery (%)
fish meat sausage	25.0	96
	12.5	107
	6.3	114
	3.1	120
freeze-dried egg soup	29.8	124
	14.9	124
	7.4	125
	3.7	141
chicken meatball	25.0	97
	12.5	100
	6.3	105
	3.1	85

Table 4. Intra- and Interassay Variances in the Sandwich ELISA Method Using Three Model Processed Foods[a]

sample	concentration (mg/kg)	intra-assay	interassay
fish meat sausage	10	2.8	5.3
freeze-dried egg soup	11.9	2.3	6.2
chicken meatball	10	2.8	8.4

[a] The intra-assay variances were calculated from 10 replicates of the same extract, and the interassay variances were calculated from triplicate analysis of the same extract on 10 different days.

Application to the Commercial Food Products. Thirty-two different commercial food samples were analyzed by the sandwich ELISA method. Each commercial food was homogenized, and the extracts were obtained according to the extraction procedure described in the Materials and Methods section. As shown in **Table 5**, 15 commercial foods with a label of shrimp or crab on the ingredients list were clearly detected. In contrast, the levels in products without a label of shrimp or crab on the ingredients list were detected to be less than 1.0 mg/kg. There were no false positives from the no-declaration samples and no false negatives from the declaration samples analyzed in this study. When commercial food products containing shrimp or crab were serially diluted and assayed, each sample gave results close to linearity (r^2 = 0.993–1.000), confirming parallelism between the calibrators and the food samples. These results show that the sandwich ELISA method could appropriately determine the crustacean protein in the processed foods.

DISCUSSION

We established the sandwich ELISA method for the detection of crustacean protein that has a specific reactivity to the

Table 5. Analysis of Various Commercial Food Samples for Using the Sandwich ELISA Method

sample	declaration	substance	quantitative (mg/kg)	regression (r^2)
bean jammed	+	crab	264	0.998
seafood curry	+	shrimp	1780	0.999
beef curry	−		<1.0	
base of pilaf	+	crab	1100	1.000
cream pasta source	−		<1.0	
croquette	+	crab	404	1.000
croquette	−		<1.0	
croquette	−		<1.0	
dumpling	+	shrimp	77000	0.995
dumpling	+	crab	1040	1.000
dumpling	−		<1.0	
base of fried rice	+	shrimp	653	0.993
base of risotto	+	crab	36.7	0.998
spray-dried soup	−		<1.0	
gratin	+	shrimp	22400	0.995
gratin	−		<1.0	
snack	+	shrimp	100	0.998
cookie	−		<1.0	
Japanese rice cookie	−		<1.0	
fried food (prawn)	+	shrimp	282000	0.995
fried food (chicken)	−		<1.0	
fried food (poke)	−		<1.0	
fried food (oyster)	−		<1.0	
fried food (squid)	−		<1.0	
noodle	+	shrimp	145000	0.998
noodle	−		<1.0	
Japanese wheat noodle	−		<1.0	
steamed fish paste	+	crab	176	0.999
steamed fish paste	−		<1.0	
fried fish paste	+	shrimp	46.4	0.995
terrine	+	shrimp	1560	0.997
fish sausage	−		<1.0	

Decapoda group in Crustacea and applied this method to processed food. Jeoung et al. (*23*) already reported a determination method for tropomyosin. However, the cross-reactivity to mollusks and the application to processed foods have not yet been sufficiently clarified. Therefore, the reactivity and specificity of the sandwich ELISA method were tested using extracts from various Crustacea, mollusks, and commercial foods. In the test of all of the molluskan sample extracts, the reactivity levels were extremely low. The house dust mite was reported to cross-react with crustacean allergens (*24*). However, the monoclonal antibodies as the capture antibody do not cross-react with the house dust mite in the Western blot analysis (data not shown). These results suggest that this method would be specific to the Crustacea protein. However, the possibility of a cross-reaction with other less commonly used mollusks or other ingredients, such as crustacean extractants as seasonings, cannot be excluded and remains to be examined. It will be necessary to clarify the applicability of the present method.

The reactivities of lobster and prawn are similar to those of the black tiger prawn. Those of the crab group appear to be lower than those of the black tiger prawn. However, the purified swimming crab tropomyosin showed a high reactivity (154%). These results suggest that the variety of reactivities among the Decapoda group may be involved in the difference of the tropomyosin contents in the sample extracts.

Furthermore, we found that the addition of porcine skeletal tropomyosin to the sample dilution buffer in the sandwich ELISA method can appropriately determine the crustacean protein in processed foods without any food matrix effects.

As described in the Results section, the sample extracts of the model processed foods were serially diluted and assayed using the tentatively constructed sandwich ELISA method, and

a good linearity could not be observed ($r^2 = 0.936$–0.995). We considered that this result would be due to food matrix effects. Therefore, to improve the dilution linearity, we attempted to add the PTM to the sample dilution buffer. Consequently, the dilution linearity for model processed food was significantly improved by the addition of the PTM to the sample dilution buffer ($r^2 = 0.996$–0.999). The addition of troponin or actin failed to improve the dilution linearity (data not shown). These results suggest that tropomyosin may be involved in the food matrix effects, although the food matrix effect mechanism remains unclear. This method offers a new perspective for the determination of various proteins in processed food and is expected to be extremely useful in other protein-measuring methods using ELISA.

To evaluate the sandwich ELISA method for the determination of crustacean protein in processed foods, a recovery study and intra- and interassays were tested using model processed foods. The results of the analysis show that this method has a good accuracy and precision. The sandwich ELISA method's sensitivity was 0.71 ng/mL, corresponding to the 0.29 μg crustacean protein/g food sample weight. This result indicates that the sandwich ELISA method is suitable for detection in the presence of hidden crustacean protein in processed foods.

In conclusion, this sandwich ELISA method is shown to have an acceptable accuracy and precision and no false positive or false negative. This method has been demonstrated to be suitable for the quantitative measurement of the specific crustacean protein in processed foods without food matrix effects.

LITERATURE CITED

(1) Jansen, J. J.; Kardinaal, A. F.; Huijbers, G.; Vlieg-Boerstra, B. J.; Martens, B. P.; Ockhuizen, T. Prevalence of food allergy and intolerance in the adult Dutch population. *J. Allergy Clin. Immunol.* **1994**, *93*, 446–456.

(2) Bousquest, J.; Bjorksten, B.; Bruijnzeel-Koomen, C. A.; Hugget, A.; Ortolani, C.; Warner, J. O.; Smith, M. Scientific criteria and the selection of allergenic foods for product labeling. *Allergy* **1998**, *53*, 37–21.

(3) Burks, W.; Bannon, G. A.; Sicherer, S.; Sampson, H. A. Peanut-induced anaphylactic reactions. *Int. Arch. Allergy Immunol.* **1999**, *119*, 165–172.

(4) Bock, S. A.; Atkins, F. M. The natural history of peanut allergy. *J. Allergy Clin. Immunol.* **1989**, *83*, 900–904.

(5) Furlong, T. J.; DeSimone, J.; Sicherer, S. H. Peanut and tree nut allergic reactions in restaurants and other food establishments. *J. Allergy Clin. Immunol.* **2001**, *108*, 867–870.

(6) Hefle, S. L.; Nordlee, J. A.; Taylor, S. L. Allergenic foods. *Crit. Rev. Food Sci. Nutr.* **1996**, *36*, S69–89.

(7) Ebisawa, M.; Ikematsu, K.; Imai, T.; Tachimoto, H. Food allergy in Japan. *J. World Allergy Org.* **2003**, *15*, 214–217.

(8) Ebisawa, M. Epidemiology of food allergy (its comparison between Japan and other countries). *Arerugi* **2007**, *56*, 10–18.

(9) Bock, S. A.; Atkins, F. M. Patterns of food hypersensitivity during sixteen years of double-blind, placebo-controlled food challenges. *J. Pediatr.* **1990**, *117*, 561–567.

(10) Sicherer, S. H.; Furlong, T. J.; Maes, H. H.; Desnick, R. J.; Sampson, H. A.; Gelb, B. D. Genetics of peanut allergy: A twin study. *J. Allergy Clin. Immunol.* **2000**, *106*, 53–56.

(11) Sampson, H. A.; Metcalfe, D. D. Food allergies. *J. Am. Med. Assoc.* **1992**, *268*, 2840–2844.

(12) Foucard, T.; Malmheden, Y. I. A study on severe food reactions in Sweden—Is soy protein an underestimated cause of food anaphylaxis? *Allergy* **1999**, *54*, 261–265.

(13) Matsuda, R.; Yoshioka, Y.; Akiyama, H.; Aburatani, K.; Watanabe, Y.; Matsumoto, T.; Morishita, N.; Sato, H.; Mishima, T.; Gamo, R.; Kihira, Y.; Maitani, T. Interlaboratory evaluation of two enzyme-linked immunosorbent assay kits for the detection of egg, milk, wheat, buckwheat, and peanut in foods. *AOAC Int.* **2006**, *89*, 1600–1608.

(14) Nagpal, S.; Rajappa, L.; Metcalfe, D. D.; Subba Rao, P. V. Isolation and characterization of heat-stable allergens from shrimp (*Panaeus indicus*). *J. Allergy Clin. Immunol.* **1989**, *83*, 26–36.

(15) Ishikawa, M.; Shimakura, K.; Nagashima, Y.; Shiomi, K. Isolation and Properties of allergenic proteins in the Oyster *Crassostrea gigas*. *Fish. Sci.* **1997**, *64*, 610–614.

(16) Miegel, A.; Kobayashi, T.; Maeda, Y. Isolation, purification and partial characterization of tropomyosin and troponin subunits from the lobster tail muscle. *J. Muscle Res. Cell Motil.* **1992**, *13*, 608–618.

(17) Suma, Y.; Ishizaki, S.; Nagashima, Y.; Lu, Y.; Ushio, H.; Shiomi, K. Comparative analysis of barnacle tropomyosin: divergence from decapod tropomyosins and role as a potential allergen. *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.* **2007**, *147*, 230–236.

(18) Greaser, M. L.; Gergely, J. Reconstitution of troponin activity from three protein components. *J. Biol. Chem.* **1971**, *246*, 4226–4233.

(19) Bailey, K. Tropomyosin: A new asymmetric protein component of the muscle fibril. *Biochemistry* **1948**, *43*, 271–279.

(20) Kohler, G.; Milstein, C. Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. *Eur. J. Immunol.* **1976**, *6*, 511–519.

(21) Watanabe, Y.; Aburatani, K.; Mizumura, T.; Sakai, M.; Muraoka, S.; Mamegosi, S.; Honjoh, T. Novel ELISA for the detection of raw and processed egg using extraction buffer containing a surfactant and a reducing agent. *J. Immunol. Methods* **2005**, *300*, 115–123.

(22) Hourihain, J. O. B.; Kilburn, S. A.; Nordlee, J. A.; Hefle, S. L.; Taylor, S. L.; Warner, J. O. An evaluation of the sensitivity of subjects with peanut allergy to very low dose of peanut protein: A randomized, double-blind, placebo-controlled food challenge study. *J. Allergy Clin. Immunol.* **1997**, *100*, 596–600.

(23) Jeoung, B.; Reese, G.; Hauck, P.; Oliver, J. B.; Daul, C. B.; Leher, S. B. Quantification of the major brown shrimp allergen Pen a 1 (tropomyosin) by a monoclonal antibody-based sandwich ELISA. *J. Allergy Clin. Immunol.* **1997**, *100*, 229–234.

(24) Witteman, A. M.; Akkerdass, J. H.; Leeuwen, J.; Zee, J. S.; Aalberse, R. C. Identification of a cross-reactive allergen (presumably tropomyosin) in shrimp, mite and insects. *Int. Arch. Allergy Immunol.* **1994**, *105*, 56–61.

Received for review May 28, 2007. Revised manuscript received September 3, 2007. Accepted September 18, 2007. This study was supported by the Health and Labor Sciences Research Grants for Research on Food Safety from the Ministry of Health, Labor, and Welfare of Japan.

JF0715471