FOOD STANDARDS Australia New Zealand
Te Mana Kounga Kai – Ahitereiria me Aotearoa

Supporting Document 1

RISK AND TECHNICAL ASSESSMENT REPORT (APPROVAL)

Executive Summary

Advantame is a new high intensity, non-nutritive sweetener that is approximately 100 times sweeter than Aspartame and 20,000 times sweeter than sucrose. It is proposed for use in table top sweeteners and various powered or liquid drinks (e.g. fruit drinks, flavoured milks).

This risk and technical Assessment was undertaken to: (1) determine whether Advantame can deliver the intended technological function in the final food; (2) evaluate the toxicity of Advantame and establish an acceptable daily intake (ADI); and (3) compare the estimated levels of intake of Advantame with the ADI to ascertain the dietary risk to consumers.

The proposed use of Advantame as an intense sweetener in a number of foods has been determined by FSANZ to be technologically justified. It can be used in a wide range of beverages that would otherwise contain sugar and is effective as a high intensity sweetener. Additionally, at low concentrations it has a flavour enhancing effect. Advantame has been demonstrated to be stable for twelve months when used in powdered beverage mixes and thirty-six months when used in a tabletop sweetener powder.

The toxicological database for Advantame is comprehensive and consists mainly of Good Laboratory Practice (GLP) and OECD test guideline compliant studies conducted in laboratory animals (mice, rats, rabbits and dogs) and humans. The database was considered adequate to characterise the toxicological hazard of Advantame and establish an ADI for dietary risk assessment purposes.

FSANZ has independently evaluated the submitted toxicity studies on Advantame including studies on kinetics, metabolism, acute toxicity, repeat-dose toxicity, genotoxicity, immunotoxicity, reproductive toxicity and developmental toxicity. Four human studies were also evaluated.

In vitro data suggesting that Advantame was predominantly hydrolysed to Advantame-acid in the small intestine prior to absorption were confirmed in laboratory animal and human studies. The extent of conversion of Advantame to Advantame-acid in the digestive tract [usually estimated by comparing the area under the plasma concentration-time curve (AUC) for Advantame with the AUC for Advantame-acid] was at least 90% in rodents (mice, rats), 97% in rabbits and 99-100% in dogs. However, these estimates for rodents and rabbits can be considered to be the lowest level of conversion because of the analytical uncertainty surrounding the concentration of unchanged Advantame-acid in plasma. Extensive conversion of Advantame to the acid form in the gastrointestinal tract of all species tested is further supported by the absence of any unchanged Advantame in the urine and faeces of rodents or dogs following oral administration. Although relatively small oral doses have been given to humans, the conversion was also estimated to be >99%. These findings indicate that there is limited systemic exposure to parent Advantame following ingestion.

Bioavailability[1] of radiolabelled Advantame-acid was estimated to be 7-9% in rats and 8-15% in dogs based on a consideration of $AUC_{oral}/AUC_{IV.}$ There was also reasonable agreement with an estimate of bioavailability of ~10% in rats and dogs derived from measured urinary concentrations of radioactivity following oral dosing and the proportion of radioactivity present in faeces following intravenous dosing. Based on urinary radioactivity, bioavailability in humans was estimated to be at least 6%. There are no bioavailability data for rabbits. In rats, the maximum plasma concentration (C_{max}) of radiolabelled Advantame-acid and other metabolites occurred in 15-45 min while in humans the C_{max} was 1.25 h. In contrast, the maximum plasma concentration of radiolabelled Advantame-acid and other metabolites in dogs was observed at 6-8 h. The apparent elimination half-life of radioactivity from rat, dog and human plasma was 8, 95 and 4 hours, respectively. The relatively long elimination half-life of radioactivity in dogs is suggestive of enterohepatic recirculation of Advantame-acid and a number of other metabolites. A comparison of AUC values between rats and dogs at an equivalent oral dose and normalised for body surface area indicated that systemic exposure to Advantame-acid and its metabolites is approximately 40-times higher in dogs than rats.

In rats, excretion of absorbed radioactivity was predominantly via the faeces (75%), with the remainder via the urine. In dogs, the absorbed dose of radioactivity was found in approximately equal proportion in urine and faeces. There was no evidence that Advantame or any of its metabolites accumulated in any tissue or crossed the placenta. At low oral doses (5-150 mg/kg bw), absorbed Advantame-acid was extensively metabolised in rats and dogs.

The acute toxicity in rats was assessed as being very low (no deaths at 5000 mg/kg bw). No toxicologically-significant effects occurred in the majority of laboratory animal studies following repeated dietary exposure up to approximately 9300, 6500 and 2500 mg/kg bw/day in mice, rats and dogs, respectively. In rats, transient reductions in bodyweight gain occurred at the highest dose, which is likely to be attributable to the absence of an iso-caloric diet when 5% Advantame was present rather than any direct toxicological effect. The only evidence of compound-related toxicity was in a rabbit developmental study, where deaths and clinical signs occurred in dams at and above 1000 mg/kg bw/day. There was no evidence that Advantame was genotoxic or carcinogenic. There was no effect on reproduction or fetal development in rats or rabbits.

In human studies, doses up to 0.5 mg/kg bw were well-tolerated by volunteers with and without type-2 diabetes following a single dose or repeated dosing for up to 12 weeks.

An ADI has been set at 5 mg/kg bw/day, by applying a 100-fold safety factor to the no observed adverse effect level (NOAEL) of 500 mg/kg bw/day in a rabbit developmental toxicity study. The NOAEL was based on maternotoxicity at the next higher dose of 1000 mg/kg bw/day.

Comparisons of the dietary exposure to Advantame with the ADI of 5 mg/kg bw indicated that for all groups of Australian and New Zealand consumers assessed (including children), estimated dietary exposures were well below this safe level of exposure. On this basis, there are no public health and safety issues associated with the proposed addition of Advantame to food.

[1] The proportion of Advantame-acid that reaches the systemic circulation

Abbreviations

Time		**Weight**	
sec	Second	bw	Bodyweight
min	Minute	wt	Weight
d	Day	ng	Nanogram
wk	Week	µg	Microgram
mo	Month	mg	Milligram
yr	Year	kg	Kilogram
Length		**Dosing**	
nm	Nanometre	iv	Intravenous
µm	Micrometre	po	Oral
mm	Millimetre	mg/kg bw/day	mg/kg bodyweight/day
cm	Centimetre		
m	Metre		
Volume		**Concentration**	
µL	Microlitre	M	Molar
mL	Millilitre	ppb	Parts per billion
L	Litre	ppm	Parts per million
		w/v	Weight per volume
		v/v	Weight per weight

Haematology & Clinical Chemistry	
APTT	Activated partial thromboplastin time
BUN	Blood urea nitrogen
CPK	Creatinine phosphokinase
Hct	Haematocrit
Hb	Haemoglobin
HDL	High density lipoprotein
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
LUC	Large unstained cells
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean cell volume
OCT	Ornithine carbamyl transferase
PT	Prothrombin time
SGPT	Serum alanine aminotransferase
SGOT	Serum aspartate aminotransferase
Pharmacokinetics	
AUC	Area under the plasma concentration-time curve
AUC_{24}	AUC for a 24 h period
AUC_t	AUC up to the last sampling time when the mean concentration was quantifiable
CL	Total clearance
C_{max}	Maximum concentration
C_{min}	Minimum concentration
F	Bioavailability
T_{max}	Time to maximum concentration
T_{min}	Time to minimum concentration
$t_{1/2}$	Terminal half-life
λ_2	Terminal rate constant
V_{ss}	Volume of distribution at steady state

Anatomy	
GIT	Gastro-intestinal tract
Chemistry	
^{14}C	Carbon-14
CMC	Carboxymethyl cellulose
Con A	Concanavalin A
DMSO	Dimethyl sulfoxide
LC-MS/MS	Liquid chromatography tandem mass spectrometry
HPLC	High pressure liquid chromatography
LSC	Liquid scintillation counting
RH	Relative humidity
uv	Ultraviolet
Terminology	
ADI	Acceptable daily intake
ADME	Absorption, distribution, metabolism and elimination
BMI	Body mass index
ECG	Electrocardiogram
FOB	Functional observational battery
GCP	Good Clinical Practice
GLP	Good Laboratory Practice
LOAEL	Lowest observed adverse effect level
LOQ	Limit of quantification
MW	Molecular Weight
NOAEL	No observed adverse effect level
QA	Quality assurance
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid

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1. Introduction

1.1 Background

On the 18th August 2009, Food Standards Australia New Zealand (FSANZ) received an Application from Ajinomoto Company Inc. (Ajinomoto) seeking an amendment to Standard 1.3.1 – Food Additives of the *Australia New Zealand Food Standards Code* (the Code) to permit the addition of a new intense (non-nutritive) sweetener, Advantame, to a range of foods.

Advantame {N-[*N*-[3-(3-hydroxy-4-methoxyphenyl) propyl-α-aspartyl]-L-phenylalanine 1-methyl ester, monohydrate; CAS No. 714229-20-6} is an *N*-substituted derivative of Aspartame that is structurally similar to Neotame {N-[*N*-(3,3-dimethylbutyl(-L-α-aspartyl]-L-phenylalanine 1 methyl ester}. Advantame is approximately 100 times sweeter than Aspartame and 20,000 times sweeter than sucrose.

The proposed uses of Advantame are in table top sweeteners, powdered fruit drinks, instant teas, instant coffee drinks, milk and non-milk based meal replacements and protein drinks, and powdered flavoured milk and milk drinks. Ajinomoto has stated that foods sweetened with Advantame are likely to be marketed as energy-reduced foods.

1.2 Risk Assessment Questions & Scope

Advantame is a new food additive and therefore a comprehensive premarket safety assessment is required.

For this Application, the risk assessment questions were developed in the context of the Section 18 Objectives of the *Food Standards Australia New Zealand Act 1991*, having regard to the Ministerial Policy Guidelines for the addition of substances other than vitamins and minerals.

The following risk assessment questions are addressed in this Risk and Technical Assessment Report:

- Has the stated purpose for adding Advantame been articulated clearly?
- Are the amounts proposed to be added consistent with achieving the technological function?
- Is Advantame proposed to be added in a quantity and form which is consistent with achieving the stated purpose?
- Is there a need to establish a reference health standard for Advantame in order to protect public health and safety? If so, what should this be?
- If Advantame enters the food supply would the resulting exposure for all consumers pose an unacceptable risk for public health and safety?

This Risk and Technical Assessment Report is structured to address the above questions in order and comprises the following components:

(1) Food Technology Assessment, which considered whether Advantame can deliver the intended technological function in the final food and describe the chemical properties of the compound.

(2) Hazard Assessment, which evaluated the intrinsic toxicity of Advantame and considered the need to establish an appropriate reference health standard [such as an

acceptable daily intake (ADI)] to be used as the comparator in the dietary modelling.

(3) Dietary Exposure Assessment (DEA), which compared the estimated levels of intake of Advantame with the reference health standard or other appropriate comparator.

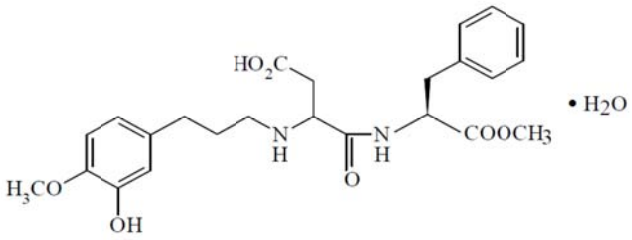
Based on these three assessment components, the risk to public health and safety in the context of the technological function of Advantame has been characterised.

2. Food Technology Assessment

2.1 Chemistry of Advantame

Advantame, also referred to as ANS9801 (a laboratory code name), is an *N*-substituted (aspartic acid portion) derivative of Aspartame that is similar in structure to neotame, which is another *N*-substituted Aspartame derivative.

2.1.1 Chemical structure & identity



CAS registry Number:	714229-20-6
Molecular Formula:	$C_{24}H_{30}N_2O_7.H_2O$
Molecular Weight:	476.52 (monohydrate)
IUPAC Name:	N-[*N*-[3-(3-hydroxy-4-methoxyphenyl) propyl-α-aspartyl]-L-phenylalanine 1-methyl ester, monohydrate
CA Name:	L-phenylalanine, N-[3-(3-hydroxy-4-methoxyphenyl)propyl]-L-alpha-aspartyl-, 2-methyl ester, monohydrate
Marketing name:	Advantame

2.1.2 Physical and chemical properties

Physical and chemical properties of Advantame were determined in a series of 18 parameters studies by the Applicant's pharmaceutical research laboratories and an analytical contractor company, the Institute of Applied Medicine. The studies were conducted in compliance with Reliability Criteria of Application Data [Article 18-4-3, Enforcement Regulations of the Pharmaceutical Affairs Law (MHW Ordinance No. 1, 1961)]. Almost all the determinations were carried out in 3 replicates and in many cases, at different temperatures and over a range of concentrations. Validation of analytical procedures and the test method for Advantame was supplied by the Applicant to the contractor company for quantifying Advantame. Some of the results are summarised in Table 2.1.

TABLE 2.1: PHYSICAL & CHEMICAL PROPERTIES OF ADVANTAME

Chemical/physical parameter		References
Melting point (°C)	101.5	Nagahisa (2004a)
Solubility (g/dL), after 30 min at 15°C; 60°C, in water: in ethanol: in ethyl acetate	 0.076; 0.586 0.798; 32.277 0.165; 2.271	Kato (2004a)
Dissolution rate[2] (%) at 37°C after: 1 min 3 min 5 min	38.5 48.0 54.7	Kato (2004b)
pH*	6.25	Nagahisa (2004a)
Dissociation constant (pKa_1; pKa_2)	2.94; 7.96	Nagahisa (2005a)
Octanol/water partition coefficient[3]; Log *P*	1.94; 0.289	Kato (2004c)
Refractive index*	1.3332	Kato (2004d)
Specific gravity*	1.0008	Kato (2004e)
Viscosity*, mPa.s	1.10	Kato (2004f)

*at 0.0005% Advantame (w/v) in water

2.1.3 Methods of analysis

The assay for Advantame and the validation of this method is presented in full detail in the Application. This method employs HPLC coupled with an ultraviolet (UV) absorption detector. Briefly, the samples are dissolved in a solution of 70% water: 30% acetonitrile, then separated using the Inertsil ODS-2 column and analysed by UV detection at 210nm. Benzoic acid and Advantame dissolved in the same mixture of water and acetonitrile are employed as the internal standard and the reference standard solutions, respectively.

The HPLC method employed in the analysis of the Advantame also quantifies Advantame-acid (a breakdown product of Advantame; refer Figure 2.1)[4] and other related substances (see section 2.1.4) in tabletop sweeteners and powdered beverages. A calibration curve based on 5 standard Advantame or Advantame-acid solutions is used.

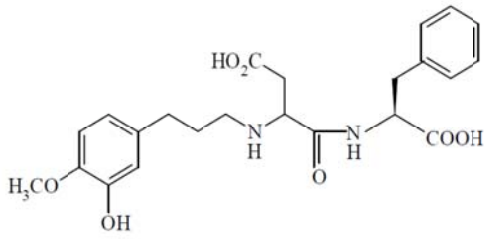


FIGURE 2.1: CHEMICAL STRUCTURE OF ADVANTAME-ACID

2.1.4 Identity and purity of Advantame

In establishing the physical chemical properties of Advantame, the determination of the purity and specificity of Advantame was carried out by the Applicant's pharmaceutical research laboratories and an analytical contractor company, the Institute of Applied Medicine. Each of these tests included validation reports that have demonstrated consistency and accuracy (Saizuka 2001; Kato 2004g,h,i,j,k & l; Nagahisa 2004c,d,e,f,g &

[2] Advantame is added at 50 mg to 900 mL of water and is expressed as dissolution ratio to the added amount of Advantame.
[3] The partition coefficient is the ratio of concentrations of un-ionised compound between two solvents. The log of the partition coefficient in the solvents is called log *P*.
[4] N-[*N*-[3-(3-hydroxy-4-methoxyphenyl)propyl-α-aspartyl]-L-phenylalanine (CAS Registry Number: 713524-95-9)

2005b).

The analytical information for 6 non-consecutive batches of Advantame, including analysis of identity, impurities (related substances) and contaminants, such as heavy metals, lead, arsenic, residual solvents microbial contaminants were provided to FSANZ by the Applicant. These demonstrated that their product complies with the specification in Table 2.2.

The Applicant provided HPLC analyses of Advantame samples showed that there were 27 identifiable peaks. Apart from ANS9801-acid, these are described as the 'total related substances' and totalled less than 1.5% of the Advantame".[5] In addition to ANS9801-acid, only four substances were present above 0.1% and are shown in Figure 2.2.

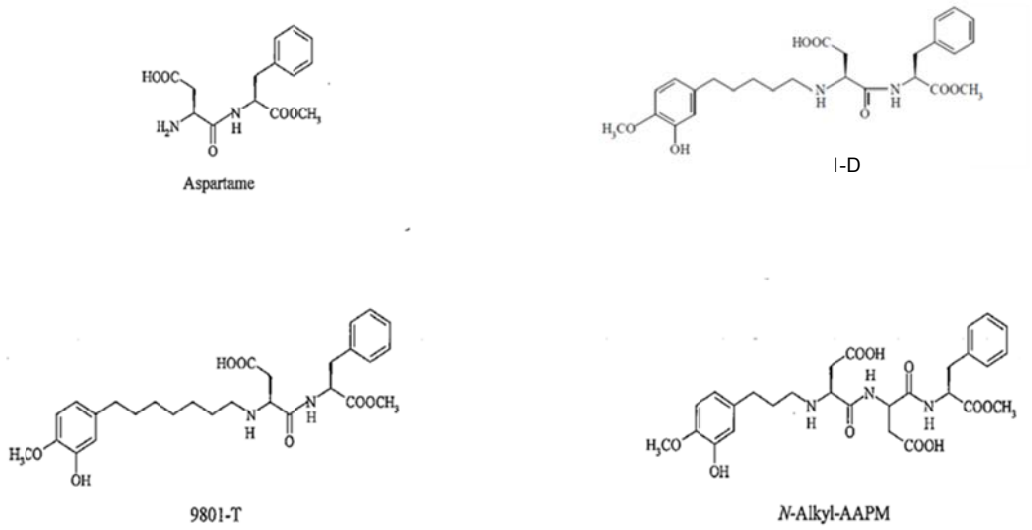


FIGURE 2.2: CHEMICAL STRUCTURE OF KNOWN RELATED SUBSTANCES
9801-D = N-[*N*-[3-(3-hydroxy-4-methoxyphenyl) pentyl]-α-L-aspartyl]-L-phenylalanine 1-methyl ester
9801-T= N-[*N*-[3-(3-hydroxy-4-methoxyphenyl) heptyl]-α-L-aspartyl]-L-phenylalanine 1-methyl ester
N-alkyl-AAPM = N-[N-[*N*-[3-(3-hydroxy-4-methoxyphenyl)propyl]-α-L-aspartyl]-α-L- aspartyl]-L-phenylalanine 1-methyl ester

2.2 Manufacture

Advantame is synthesised from Aspartame and 3-(3-hydroxy-4-methoxyphenyl)-propionaldehyde (HMPA) in a one step process by reductive N-alkylation, which is carried out by treating Aspartame and the aldehyde with hydrogen in the presence of a platinum catalyst (Pt/C) in a methanolic solution (Amino et al 2008). The temperature is raised and the resulting slurry undergoes reductive N-alkylation to produce Advantame (Figure 2.3). The reaction mixture is filtrated to remove the reaction catalysts and concentrated under reduced pressure. To extract the Advantame from the remaining filtrate, methanol and isopropyl acetate are added and the temperature is reduced to crystallize the Advantame, which is then removed and purified.

[5]According to the Applicant, several related substances of Advantame have been identified in the final Advantame product as manufacturing impurities. HPLC analysis of 12 production lots revealed 27 substances (detection limit 0.01%) related to Advantame and of these, only 16 peaks were quantifiable (0.02%). The remaining related substances were found only in trace amounts in the sample lots or were not detected at all in some samples. Of the 16 quantifiable peaks, only five were present in the final Advantame material at levels above 0.1%. These substances were fully characterised. Eleven other substances were present in the product in quantifiable amounts, but at levels below 0.1%.

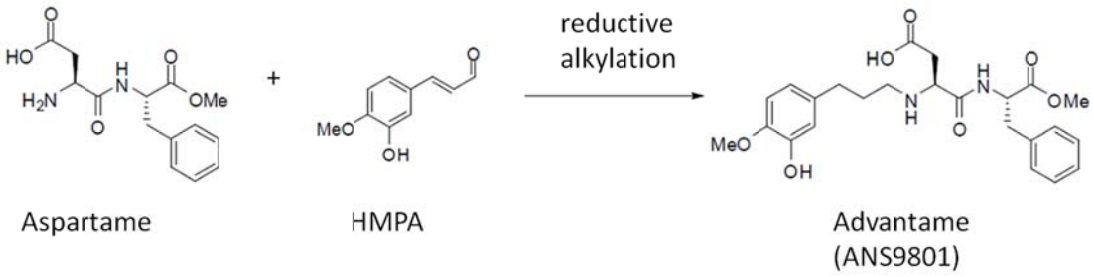


FIGURE 2.3: REACTION PATHWAY FOR SYNTHESIS OF ADVANTAME (MODIFIED FROM KAWAHARA ET AL 2007)

2.2.1 Advantame Specifications

Advantame is not covered by a specification from one of the published sources identified in Standard 1.3.4 – Identity and Purity or in any of the primary or secondary specification prescribed in Standard 1.3.4.. In the absence of an appropriate published monograph, a detailed specification was provided by the Applicant and is presented in Table 2.2.

TABLE 2.2: PRODUCT SPECIFICATIONS FOR ADVANTAME

Specification Parameter	Specification Value	Analytical Methodology
Identification		
IR absorption spectrum	As Reference standard provided by Ajinomoto Co.	IR Spectrophotometry with potassium bromide disk
Purity		
Assay	Not less than 97.0% and not more than 102.0% on anhydrous basis	HPLC internal standard method
Specific rotation $[\alpha]^{20}$ D	Between -45^0 and -38^0	Japanese Pharmacopeia method
ANS9801-acid	Not more than 1.0%	HPLC method
Total other related substances	Not more than 1.5%	HPLC method
Water	Between 2.5 and 5.0%	Karl Fischer coulometric titration
Residue on ignition	Not more than 0.2%	Japanese Pharmacopeia method
Total Heavy Metals (mg/kg)	Not more than 40[6]	Japanese Pharmacopeia method
Lead (mg/kg)	Not more than 2	Atomic absorption
Arsenic (mg/kg)	Not more than 1	Japanese Pharmacopeia method
Residual Solvents (mg/kg)		
Methyl Acetate	Not more than 500	Gas chromatography
Isopropyl Acetate	Not more than 2,000	Gas chromatography
Methanol	Not more than 500	Gas chromatography
2-Propanol	Not more than 500	Gas chromatography

2.3 Technological justification

The Applicant proposes that Advantame be used in foods to provide high intensity sweetness while maintaining their flavour integrity. In addition, when used at a low level,

[6] The limits for total heavy metals, lead and arsenic are as per clause 4 of Standard 1.3.4 – Identity and Purity

Advantame also enhances existing flavour of food. The Applicant has carried out laboratory sensory studies examining the effect of using Advantame as a sweetener in several food types in which it is proposed for use.

There are limited peer-reviewed published technological data on Advantame. The Applicant has provided seven major laboratory studies in their Application dossier to demonstrate the technological functions and stability of Advantame. Some of their studies are summarised below.

2.3.1 Technological justification as an intense sweetener

Advantame was demonstrated to perform "very well" as a sweetener in coffee (hot and warm), iced tea, and powdered beverage formulations. The sensory results on the amount of Advantame required to achieve different sweetness levels in beverages are summarized in Table 3.

TABLE 2.3: SENSORY RESULTS OF SWEETNESS LEVELS IN FOOD PRODUCTS CONTAINING ADVANTAME.

Reconstituted food products	Typical ("just about right") level (mg/L)	Min–maximum level tested (mg/L)
Iced tea (Willis 2005a)	3.0	0.5-10.0
Hot coffee* (Willis 2005b)	1.8	Not available
Instant iced coffee	3.1	0.6-6.4
Powdered beverage mixes** (Sakata 2007a)	4.0	1.0-12.0

*not significantly different from warm coffee; **a lemonade-flavoured powdered beverage was used; Note: the coffee and teas tested did not contain milk

A series of sensory evaluations at various concentrations in water compared to Aspartame sweetened solutions demonstrated that the sweetness potency of Advantame was approximately 70 to 120 times greater than Aspartame, varying with sweetener concentration and its application in water, food, beverages and as a tabletop sweetener (Willis 2005c & d; Sakata 2005). Furthermore, Advantame was demonstrated to have a similar sensory profile to Aspartame, especially at high concentrations with a dominant sweet flavour, while perceived intensities for bitter and sour flavour seemed to be suppressed.

2.3.2 Technological justification as a flavour enhancer

Advantame has flavour enhancement effects in foods such as lemon tea, orange juice and strawberry yoghurt at very low concentrations (Amino et al 2008). According to Schedule 5 of Standard 1.3.1 of the Australia New Zealand Food Standards Code (The Code), a flavour enhancer is a substance added to enhance the existing taste and/or odour of a food. The flavour enhancing properties of Advantame were evaluated by the Applicant at concentrations below its sweetening level (Willis 2005e). A GDTL [7] (Group difference threshold level) test was used to determine the range of concentrations of Advantame that provide flavour enhancement at or below the GDLT.

For example, the GDTL of Advantame was determined to be 1.46 mg/kg using Strawberry

[7] GDTL is defined as the concentration of Advantame in 9.56% sucrose solution that corresponds to the median *individual difference threshold*.
The difference threshold for an individual person is defined as the highest concentration of Advantame in a 9.56% sucrose solution that was not noticeably sweeter than the 9.56% sucrose solution (without Advantame).

Kool-Aid® dissolved in water with a resulting concentration of 9.56% sucrose, with flavour enhancement properties at levels between 1.0-1.46 mg/kg of finished drink.

2.3.3 Anticipated Food Uses

Since Advantame is a high intensity sweetener, approximately 20,000 times sweeter than sucrose and 100 times sweeter than Aspartame, the use level of Advantame will be lower than the current use level of added sweeteners.

The food groups proposed by the Applicant to contain Advantame are presented in Table 2.4.

TABLE 2.4: FOOD CATEGORIES AND TYPICAL USE LEVELS OF ADVANTAME AS A SWEETENER

Food Category	Proposed Food Uses	Use level (mg/L)
Non-Alcoholic Beverages*	Powdered Non-Milk Based Meal Replacements and Protein Drinks	2.9
Coffee, coffee substitutes, tea, herbal infusions and similar products*	Instant Teas	3.0
	Instant Coffee Drinks	1.8
Dairy Products*	Powdered Flavoured Milk and Milk Drinks	2.9-4.4
	Powdered Milk-Based Meal Replacements and Protein Drinks	4.4
Fruit and vegetable juice products*	Powdered Fruit Flavoured Drinks	4
Sugar Substitutes(mg/kg)	Tabletop Sugar Substitutes (powdered and tablets)	450

*Only powdered and artificially sweetened versions of these foods are proposed for use for Advantame

2.4 Stability of Advantame

In general, Advantame in dry form such as table top sweetener or powdered soft drinks mix is very stable and keeps its functionality under the usual storage conditions [25°C/60% Relative Humidity (RH)] (Amino et al 2008).

2.4.1 Stability of Advantame in bulk (Kato 2004m; Nagahisa 2004h & i; Ito 2007)

In a preliminary accelerated stability test, the Applicant evaluated the stability of Advantame produced on a laboratory scale under stressed conditions and examined and identified the degradation products. The Applicant has conducted a bulk stability study with several lots of Advantame stored at 25°C/60% RH over 60 months (5 years) with an interim report issued after 36 months. Bulk stability testing was also conducted with 3 lots of Advantame under accelerated stability testing conditions (i.e. 40°C/75% RH) for 6 months. The content of Advantame remaining, breakdown products and presence of related substances (e.g. 9801-D and 9801-T, etc) at each testing interval during stability testing were assessed using HPLC.

During storage there is a slight drop (typically 0.2%) in the Advantame content and a corresponding increase in ANS9801-acid and the total related substances due to breakdown of Advantame. The major breakdown products identified in all samples were ANS9801-acid followed by 9801-T, N-Alkyl-AAPM, Aspartame, and 9801-D in descending amounts.

In the studies, after 36 months, the content of Advantame ranged from 98.5 to 99.7%, while the content of the principal breakdown product ranged from 0.13 to 0.15%. Following 60

months of storage, the content of Advantame ranged from 98.7% to 99.4% and the amount of ANS980-acid ranged from 0.18% to 0.19%. Another major breakdown product, N-alkyl-AAPM, was demonstrated to be stable between 36 and 60 months at 0.11%. The proportion of other breakdown products grouped under “total related substances” (e.g. 9801-D and 9801-T) were 0.55 to 0.65%.

In conclusion, Advantame in dry form stored in bulk was stable under standard conditions of storage (25°C/60%RH) for up to 36 months, as well as up to 60 months stored in bulk in stability testing chambers.

2.4.2 Photostability of Advantame (Ito 2008)

The photostability of Advantame was assessed using a single lot of Advantame stored at 30°C/65% RH and exposed to light or no light for 2 weeks. The samples were placed in the stability testing area in glass Petri dishes with the control samples covered in aluminium to protect them from the light. The samples were tested at baseline, after 1 week of testing, and after 2 weeks. The results showed that Advantame in dry powder form was relatively stable in light, with no increase in breakdown products.

2.4.3 Stability and Functionality Study of Advantame in a Tabletop Sweetener Powder (Sakata 2008)

The chemical stability and functionality of Advantame (ANS9801) as a sweetener in a tabletop powder stored at 25°C/60% RH over 36 months was assessed by the Applicant. The study was designed to simulate storage of typical tabletop products, which consist of sweetened tabletop powder packed in individual paper sachets and combined in cardboard cartons. The tabletop powder was composed of Advantame diluted with dextrose and maltodextrin (blended at a ratio of 97 to 3).

The chemical stability was measured using the HPLC method, while the sensory characteristics (i.e. the functionality) of Advantame were examined in an iced coffee beverage provided to a sensory panel. The chemical stability and sensory characteristics of Advantame in the tabletop product were evaluated following 12, 25, and 36 months of storage.

The results of the study demonstrated that Advantame in the tabletop product remains stable with minimal formation of ANS9801-acid and maintains its sensory functions following 36 months of storage at 25°C/60% RH.

2.4.4 Stability and Functionality of Advantame in Powdered Beverage Mixes (Sakata 2007b)

The chemical stability and functionality of Advantame as a sweetener in powdered beverage mixes was examined for up to 12 months when stored under normal (i.e. 25°C/60% RH), intermediate (i.e. 30°C/65% RH), or accelerated storage conditions (i.e. 40°C/75% RH). The study was designed to simulate the storage of an Advantame sweetened lemon-flavoured powdered beverage mix (i.e. dry) packaged in heat sealed pouches during storage.

The sweetened powdered beverage mix was composed of Advantame, maltodextrin, citric acid, trisodium citrate, tricalcium phosphate, L-ascorbic acid, food colour (Yellow No. 4), and lemon flavour (lemon flavour was used since it is one of the most commonly used powdered beverage mix flavours). The Advantame sweetened, as well as unsweetened, powdered beverage mixes were packaged in separate heat sealed pouches composed of paper/polyethylene/aluminium/ ethylene methacrylic acid copolymer.

The chemical stability was measured using the HPLC method. The functionality of Advantame in the dry powdered beverage mix was evaluated by sensory panels composed of 15 to 17 individuals that rated the level of sweetness using the 5-point just-about-right scale.

Advantame was demonstrated to be stable and retain its functionality in the powdered beverage mix for 12 months of storage under normal conditions and 6 months under intermediate and accelerated conditions.

2.5 Conclusion

The use of Advantame as an intense sweetener and flavour enhancer in a number of foods specified by the Applicant is technologically justified.

Advantame is effective as a high intensity sweetener, 20,000 times sweeter than sucrose. Additionally, at low concentrations it has a flavour enhancing effect.

It can be used in a wide range of beverages that would otherwise contain sugar and has been demonstrated to be stable for 12 months when used in powdered beverage mixes and 36 months when used in a tabletop sweetener powder.

3. Hazard Assessment

3.1. Background

3.1.1 Chemistry

Details of the physicochemical properties of Advantame, including product specifications and the impurity profile, are included in the Food Technology Assessment (Section 2).

Low concentrations of a number of different manufacturing impurities and breakdown products are present in Advantame preparations. Toxicity studies have been conducted on the same batches of Advantame used to analyse stability and establish product specifications.

3.1.2 Assessments by Other Agencies

Advantame has not previously been assessed by any national agency or international body. The Applicant has stated that an application for the use of Advantame as a food additive is currently under review by the US Food and Drug Administration.

3.1.3 Scope of the Hazard Assessment

FSANZ has not previously assessed the safety of Advantame. Therefore, the aims of the current assessment were to:

- Evaluate all the available data on the toxicology of Advantame to assist in determining the hazard of Advantame as a novel sweetener; and

- If needed, establish a suitable reference health standard such as an acceptable daily intake (ADI) for Advantame.

3.2 Evaluation of Submitted Data

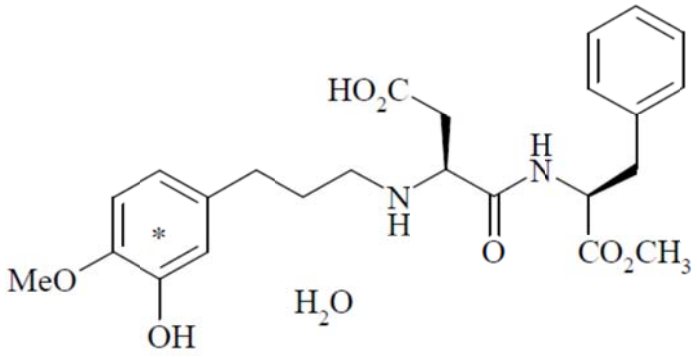
The toxicological database for Advantame is extensive and consists entirely of unpublished studies sponsored by the Applicant and conducted mainly in contract laboratories. The number of studies performed in multiple laboratory animal species (mice, rats, rabbits and dogs) and in humans provides a sound basis to assess the toxicological hazard of Advantame and establish an ADI.

FSANZ has independently evaluated the submitted toxicity studies on Advantame including studies on pharmacokinetics, metabolism, acute toxicity, repeat-dose toxicity (including carcinogenicity), genotoxicity, reproductive toxicity and developmental toxicity. These studies have been conducted according to principles of Good Laboratory Practice (GLP) and all study reports contained Quality Assurance (QA) statements. In studies conducted in humans, statements of compliance with Good Clinical Practice (GCP) were made. Unless otherwise indicated, the concentration, stability and homogeneity of Advantame in the dosing solutions or diets were established. Additionally, the majority of data were subjected to statistical analyses by the performing laboratory. In this hazard assessment, specific results from toxicity studies are only reported by exception.

Plasma concentrations of Advantame and its main metabolite, Advantame-acid, were analysed in a number of studies using a liquid chromatography tandem mass spectrometry (LC-MS/MS) assay. However, it was subsequently determined that the HCl/methanol extraction method used to prepare samples leads to re-esterification of Advantame-acid to

Advantame (maximum of 5% conversion). Consequently, the concentrations of Advantame may be overestimated (and the concentration of Advantame-acid underestimated) in these studies. In the current report, those studies using this method have been marked with an asterix (*****). While there is uncertainty about the accuracy of the results, these studies have been included in this report because they are considered to provide qualitative information on the kinetics and metabolism of Advantame in laboratory animals.

In studies where [^{14}C]- or [$^{13}C_6$]-Advantame was administered, it was labelled at the 3-hydroxy-4-methoxy phenyl moiety as follows:



3.2.1 Absorption, Distribution, Metabolism & Elimination (ADME) Studies

In vitro data evaluated as part of the current Application (see Section 3.2.2) indicated that most of an oral dose of Advantame would be converted to Advantame-acid in the small intestine. Given the consequent uncertainty surrounding the actual dose of Advantame laboratory animals and humans would be exposed to and the interspecies variability of the conversion in the small intestine, pharmacokinetic parameters given in the proceeding studies are based on total plasma radioactivity.

3.2.1.1 Rats

*****Aikens PJ, Kirkpatrick D, Pluthero MG, Kane TJ, Baldrey SF, Flack I, Shafait S & Holding JD (2004a) ANS9801. Pharmacokinetics of single doses in the rat after oral and intravenous administration. Report No. AJO 184/034042. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. **GLP**: UK, EC, OECD, Japan & US FDA. **QA statement**: Yes.

[^{14}C]-Advantame (>98% radiochemical purity; Batch No. LFE/14C-ANS/74; synthesised by the performing laboratory) was mixed with unlabelled Advantame (99.9% purity; Batch No. 000825; sourced from the Sponsor) and administered to Han Wistar (Hsd Brl Han:WIST) rats as a single gavage dose of 5 or 150 mg/kg bw in 1% (w/v) carboxymethyl cellulose (CMC) (30 rats/sex/dose), or as a single intravenous (IV) dose of 5 mg/kg bw in isotonic saline (33 rats/sex). It was stated that food was available *ad libitum* throughout the study; in the absence of any qualification in the study report, this was taken to mean that rats were not fasted prior to dosing. Three undosed rats/group served as controls. Rats were sourced from Harlan UK Ltd (Bicester, England), weighed 183-236 g and were 7-10 weeks old. Following dosing, rats were housed by sex, with food and water available *ad libitum*. At 0.1 (IV group only), 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 12 and 24 h after dosing, blood samples were collected from 3 rats/sex. The concentration of radioactivity in plasma was analysed by liquid scintillation counting (LSC), while the concentrations of Advantame and Advantame-acid were measured by liquid chromatography tandem mass spectrometry (LC-MS/MS). C_{max} and T_{max} values were recorded while the plasma concentration-time curve (AUC) was estimated using the linear trapezoidal rule. Other calculated pharmacokinetic parameters included: AUC_t (AUC up to the last sampling time when the mean concentration was quantifiable);

AUC (AUC to infinite time); λ_z (terminal rate constant); $t_{1/2}$ (terminal half-life); CL (total clearance); V_{ss} (volume of distribution at steady state); and F (bioavailability).
At the low oral dose, the concentration of Advantame in plasma was below the limit of quantification (LOQ) but was quantifiable at the high oral dose, and IV dose. However, the study authors considered that the detection of Advantame in the two latter groups was an artefact of the extraction method (as described above). In all groups, quantifiable concentrations of Advantame-acid were detectable in plasma. At the low oral dose, Advantame-acid was <LOQ from 8 h in males and 12 h in females. At the high oral dose, quantifiable Advantame-acid was still detectable in plasma at 24 h after dosing. In the IV dose group, Advantame-acid was <LOQ from 6 h after dosing.

Pharmacokinetic parameters for plasma radioactivity (i.e. the total of Advantame and its metabolites including Advantame-acid) and unlabelled Advantame-acid are presented in Table 3.1. Absorption of radioactivity was relatively rapid, with T_{max} values of 15 min at the low oral dose and 30 or 45 min at the high oral dose. Mean C_{max} and AUC values increased proportionally (i.e. ~30-fold) from the low to high oral dose, suggesting linear kinetics. The elimination half-life was 6-8 h. The oral bioavailability of radioactivity was calculated to be less than 10% of the administered radioactive dose. For unlabelled Advantame-acid, the C_{max} appeared to be proportional to dose in males. In females the C_{max} and AUC_t in both sexes showed a greater-than dose proportional increase (~50-fold increase *versus* the expected 30-fold increase). However, given the analytical uncertainty surrounding the accuracy of the unlabelled Advantame-acid assay (see Section 3.2), it is difficult to have confidence in the absence of dose-response proportionality.

While it is conventional to determine the extent of metabolism by comparing AUC_t values for Advantame-acid (the metabolite absorbed from the GI tract) with total radioactivity, this was unreliable owing to the incompatibility of the concentration estimates generated by the LC-MS/MS method with radioactivity. In this study, the concordance with an estimate derived from radioactivity excreted in urine was rather poor. The LC-MS/MS estimate of Advantame-acid in plasma accounted for approximately 4-7% and 10-11% at the low (5 mg/kg bw) and high (150 mg/kg bw) oral doses, respectively. Hence the extent of Advantame-acid metabolism was 93-96% at the low dose and 89-90% at the high oral doses. Based on urinary excretion data, the estimate for Advantame-acid metabolism was only between 50 to 75% (Aikens et al 2005e). The disparity in these results reinforces the problem identified by the investigators that resulted in underestimating the concentration of Advantame-acid using the LC-MS/MS method. This underestimation was corrected in the assay used in the long-term repeat dose studies in rats.

TABLE 3.1: PHARMACOKINETIC PARAMETERS FOR RATS

Parameter	5 mg/kg bw IV		5 mg/kg bw PO		150 mg/kg bw PO	
	Male	Female	Male	Female	Male	Female
Radioactivity[a]						
Achieved dose (mg/kg bw)	5.09	5.14	5.01	5.07	142.04	143.19
C_{max} (ng eq./g)	-	-	105	136	2366	4066
T_{max} (h)	-	-	0.25	0.25	0.75	0.5
AUC_t (ng eq.h/g)[b]	4010	3790	313	348	7760	9510
AUC_∞ (ng eq.h/g)	4330[c]	4120[c]	334	382	8010	10100
λ_z (h^{-1})	0.0441[c]	0.0470[c]	0.0964	0.0855	0.1163	0.0944
$T_{1/2}$ (h)	15.7[c]	14.7[c]	7.2	8.1	6.0	7.3

Parameter	5 mg/kg bw IV		5 mg/kg bw PO		150 mg/kg bw PO	
	Male	Female	Male	Female	Male	Female
CL (mL/min/kg)	19.3[c]	20.3[c]	-	-	-	-
V_{ss} (L/kg)	5.61[c]	6.42[c]	-	-	-	-
F (%)[d]	-	-	7.8	9.4	6.6	8.8
Advantame-acid[e]						
C_{max} (ng/g)	-	-	7.6	8.1	226.7	412.9
T_{max} (h)	-	-	0.75	0.25	1	0.5
AUC_t (ng.h/g)[b]	1480	1310	13.1	23.2	875	1010
$T_{1/2}$ (h)	0.6	0.6	1.9[c]	2.1[c]	3.1[c]	3.6[c]

Results expressed as means; a = concentrations expressed as Advantame equivalents; b = AUC up to the last sampling time at which the mean concentration was quantifiable (i.e. 24 h); c = estimate only; d = calculated from AUC values; e = analysed by a non-radioactive method

Aikens PJ, Kirkpatrick D, Pluthero MG & Kane TJ (2002a) ANS9801. Tissue distribution in the male rat. Report No. AJO 181/013583. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. **GLP**: UK, EC, OECD, Japan & US FDA. **QA statement**: Yes.

Experimental

[^{14}C]-Advantame (98.3% radiochemical purity; Batch No. LFE/14C-ANS/65; synthesised by the performing laboratory), diluted with unlabelled Advantame (99.9% purity; Lot No. 000825; sourced the Sponsor) in 1% (w/v) CMC, was administered to twenty-one male Lister Hooded (Crl:LISBR) rats (196-217 g bodyweight; 6-7 weeks old; sourced from Charles River, Kent, UK) as a single gavage dose of 5 mg/kg bw. Three rats were sacrificed at 0.25, 1, 2, 6, 12, 24 and 48 h after dosing and range of organs/tissues analysed for radioactivity by LSC.

Results

Low levels of radioactivity were detected in the majority of organs/tissues from 15 min after dosing except for the adrenals, bone marrow and thyroid gland, which contained no detectable radioactivity. The level of radioactivity in all tissues rapidly declined over the duration of the study, with the vast majority excreted by 24 h. There was no indication of any accumulation of radioactivity in any organ or tissue. The mean level of radioactivity in organs with radioactivity greater than that measured in plasma (i.e. a tissue:plasma ratio >1), over time, is given in Table 3.2. Those organs/tissues with radioactivity less than plasma included brain, eyes, fat, heart, lungs, lymph nodes, muscles, pancreas, salivary gland, skin, spleen, testes and thymus. The majority of radioactivity was detected in the contents of the GIT (~70% of the administered dose over the first 6 h), with the actual tissues of the digestive tract having the next highest levels (~23% over the first h, ~20% at 2 h and 15% at 6 h). Based on the level of radioactivity in organs/tissues other than the GIT and its contents (89.2-97.1% at 6 h), the level of gastrointestinal absorption is estimated to be 3-11% of the administered dose.

TABLE 3.2: LEVELS OF RADIOACTIVITY IN SELECTED ORGANS/TISSUES OVER TIME

Organ or tissue	Time (h)						
	0.25	1	2	6	12	25	48
Stomach contents	79.0 (50.61)	45.5 (28.38)	34.3 (18.51)	16.1 (3.703)	0.034 (0.0213)	0.284 (0.1608)	nd
Stomach	39.6 (3.913)	19.0 (2.122)	9.09 (0.9089)	5.12 (0.5258)	0.138 (0.0176)	0.110 (0.0112)	nd
Small intestine contents	34.7 (16.96)	71.1 (42.29)	92.2 (56.30)	10.8 (3.932)	0.146 (0.0977)	0.074 (0.0552)	0.004 (0.0030)

Organ or tissue	Time (h)						
	0.25	1	2	6	12	25	48
Small intestine	47.2 (19.11)	56.9 (21.54)	56.1 (19.47)	5.66 (2.047)	0.077 (0.0344)	0.059 (0.0222)	0.004 (0.0012)
Caecum contents	0.162 (0.1008)	0.222 (0.1226)	5.06 (1.170)	103 (48.26)	36.3 (19.76)	3.14 (1.556)	0.035 (0.0197)
Caecum	1.02 (0.0713)	1.27 (0.0947)	7.28 (0.5277)	91.4 (6.279)	28.4 (2.931)	2.32 (0.1625)	0.029 (0.0022)
Large intestine contents	0.214 (0.0695)	0.133 (0.0220)	0.185 (0.0975)	61.8 (16.97)	24.1 (5.616)	1.29 (0.4331)	0.028 (0.0058)
Large intestine	1.85 (0.1928)	2.53 (0.2516)	1.61 (0.1049)	75.4 (7.492)	17.9 (1.600)	0.972 (0.0831)	0.014 (0.0013)
Liver	0.246 (0.2553)	0.132 (0.1436)	0.077 (0.0785)	0.029 (0.0272)	0.010 (0.0097)	0.002 (0.0024)	nd
Kidneys	0.271 (0.0478)	0.157 (0.0272)	0.090 (0.0150)	0.036 (0.0061)	0.013 (0.0023)	0.005 (0.0009)	nd
Urinary bladder	0.346 (0.0017)	0.188 (0.0011)	0.335 (0.0018)	0.105 (0.0006)	nd	nd	nd
Prostate	0.150 (0.0017)	0.070 (0.0009)	0.043 (0.0006)	0.049 (0.0007)	nd	nd	nd
Plasma	0.098 (0.0091)	0.066 (0.0061)	0.033 (0.0031)	0.012 (0.0011)	0.003 (0.0003)	nd	nd

Results expressed as the mean µg equivalent Advantame/g tissue (n=3), with the % of the administered radioactive dose contained in parentheses; nd = not detectable

Conclusion

Following oral administration, radioactivity was rapidly absorbed from the GIT, with the level of absorption estimated at 2.9-10.8%. There was no indication of accumulation in any organ or tissue, with the majority excreted by 24 h.

Aikens PJ, Kane TJ & Houchen T (2004b) ANS9801. Determination of the distribution in rats by whole-body autoradiography. Report No. AJO 217/042246. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. **GLP**: UK, EC, OECD, Japan & US FDA. **QA statement**: Yes.

[^{14}C]-Advantame (98.8% radiochemical purity; Batch No. AJO/186/TW/43; synthesised by the performing laboratory) in 1% (w/v) CMC, was administered to pregnant and non-pregnant female, and male Han Wistar rats (5/group) as a single gavage dose of 5 mg/kg bw. Rats were sourced from Harlan UK (Oxon, England) and weighed 227-233 g (males), 182-199 g (females) or 256-285 g (pregnant). One rat/group was sacrificed at 0.25, 1, 2, 6 and 12 h after dosing and the tissue distribution of radioactivity analysed by whole-body autoradiography.

The tissue distribution of radioactivity was qualitatively similar across the three groups. The highest level of radioactivity was observed 15 min after dosing, which rapidly declined in succeeding autoradiographs, with only very low levels remaining 12 h after dosing. The highest level of radioactivity was detected in the GIT and its contents, with lower levels detected in the liver, kidneys and bladder. Lower levels still were observed throughout the rest of the body. No radioactivity was detected in the placenta or foetus and there was no evidence of accumulation.

Aikens PJ, Kane TJ & Pluthero MG (2005d) ANS9801. Metabolism in the rat preliminary investigations. Report No. AJO 172/014349. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. **GLP**: UK, EC, OECD, Japan & US FDA). **QA statement**: Yes.

Experimental

In this pilot study, a series of experiments was undertaken in male and female Han Wistar rats (Hsd Brl Han:WIST) (170-265 g bodyweight 5-7 weeks of age sourced from Harlan UK) to examine the absorption, metabolism and excretion of [^{14}C]-Advantame (≥98% radiochemical purity; Batch No. LFE/14C-ANS/65; synthesised by the performing laboratory) administered as a single gavage dose of 5 mg/kg bw in 1% w/v aqueous CMC. Details of the experiments are summarised in Table 3.3. In most cases, [^{14}C]-Advantame was mixed with unlabelled Advantame (99.9% purity; Batch No. 000825; sourced from Ajinomoto Co Inc, Tokyo, Japan). In two experiments, [$^{13}C_6$]-Advantame was also administered (100% purity; Batch No. LFE/13C-ANS/82; synthesised by the performing laboratory). It was stated that food was available *ad libitum* throughout the study; in the absence of any qualification in the study report, this was taken to mean that rats were not fasted prior to dosing. Radioactivity was measured by LSC, while metabolites were analysed using HPLC with radiochemical detection.

TABLE 3.3: EXPERIMENTAL DETAILS

Exp	Dosing & animal details	Sampling details
1	[^{14}C]- & unlabelled Advantame. 3 rats/sex.	Blood collected at 0.5, 1, 2, 4, 8, 12, & 24 h.
2	[^{14}C]-, [^{13}C]- & unlabelled Advantame. 3 rats/sex.	Urine & faeces collected at 0-6, 6-12, 12-24, 24-48, 48-72, 72-96 & 96-120 h. CO_2 collected at 0-6, 6-24, 24-48 & 48-72 h. Cage washes collected every 24 h to 120 h. GIT & carcass retained for analysis following sacrifice (120 h).
3	[^{14}C]- & unlabelled Advantame. 3 bile duct cannulated rats/sex.	Bile collected at 0-3, 3-6, 6-12, 12-24 & 24-48 h. Urine, faeces & cage washes collected at 0-12, 12-24 & 24-48 h. GIT & carcass retained for analysis following sacrifice (48 h).
4	[^{14}C]-, [^{13}C]- & unlabelled Advantame. 4 bile duct cannulated males (2 donors & 2 recipients). Pooled 0-12 h bile from the 2 donors was administered to the 2 recipients via a duodenal cannula	Bile collected from donors at 0-3, 3-6, 6-12, 12-24 & 24-48 h. Bile collected from recipients at 0-3, 3-6, 6-12, 12-24 & 24-48 h. Urine, faeces & cage washes collected from recipients at 0-12, 12-24 & 24-48 h.
5	[^{14}C]-Advantame. 4 rats/sex.	Blood collected from 2 rats/sex at 0.5 & 2 h.

Results

In Experiment 1, radioactivity was rapidly absorbed; peak plasma concentrations occurred at 0.5 h after dosing, with no radioactivity detectable from 8 h after dosing. Females tended to have higher plasma concentrations of radioactivity than males (46, 42 and 33% higher at 0.5, 1 and 2 h after dosing, respectively).

In Experiment 2, recovery of radioactivity was 90.25-93.25%, with the majority excreted in faeces (89.47±2.82 and 88.21±0.68% of the administered dose in males and females, respectively) and urine (2.60±1.25 and 1.94±0.10% of the administered dose in males and females, respectively) within 48 h. No radioactivity was detectable in expired air, the residual carcass or the GIT, with only low levels detected in the cage wash (<0.4% of the administered dose). HPLC analysis detected no Advantame in urine or faeces. The main metabolite in urine and faeces was Advantame-acid (~0.6 and 40% of the administered dose, respectively). In faeces, a second major metabolite (RF-1) was detected (20 and 33% of the administered dose in males and females, respectively), which was not detected in urine. Three minor metabolites (R1, R2 and R3) were detected in urine and faeces, which accounted for less than approximately 2 and 6% of the administered dose, respectively. A small level (0.1%) of radioactive polar material was detected in male urine only. Other uncharacterised radioactive material in urine and faeces accounted for 0.22/0.13% (males/females) and 3.6/3.07% (males/females) of the administered dose, respectively. All detected urine and faecal metabolites were stable when subjected to one freeze-thaw and storage for 28 days.

In experiment 3, mean recoveries of radioactivity were 97.23±3.95% in males and 97.89±3.47% in females. Biliary excretion peaked at 0-3 h after dosing and accounted for 6.10±4.85 and 4.83±2.08% of the administered dose in males and females, respectively, over 48 h. Urinary excretion peaked at 0-12 h after dosing and accounted for a total of 5.02±1.78 and 5.71±1.40% of the administered dose in males and females, respectively. Based on the amount of radioactivity present in urine and bile, the level of GIT absorption was approximately 11%. The highest level of radioactivity was detected in the faeces (~80% of the administered dose), peaking at 12-24 h after dosing. Small levels of radioactivity were detected in the GIT tract and its contents (~4/6% in males/females), residual carcass (~0.4/1% in males/females) and cage wash (~0.7/0.5% in males/females).

Pooled (0-48 h) bile, urine and faeces contained no Advantame. Metabolites detected in bile included RF-1 (~3/1% in males/females), R4 (~2%) and Advantame-acid (~1.5%), with unidentified metabolites accounting for approximately 0.5% of the administered dose. The types of metabolites detected in urine were consistent with those in Experiment 2 (with the exception that no polar components were detected in males); the concentrations of metabolites were approximately double that of Experiment 2. In faeces, the main metabolites were RF-1 and Advantame-acid (~31/25% and 31/17% in males/females, respectively). Polar compounds (~8%), other compounds (3/4% in males/females) and metabolites R1, R2 and R3 (total of ~6% in males and 4% in females) were also present in faeces.

In Experiment 4, approximately 2% of the oral radioactive dose was excreted in the bile of the two male donors, peaking at 3-6 h in one rat and 6-12 h in the other. Following duodenal administration to the two male recipients, the recovery of radioactivity was 96.3%, with approximately 14, 8, 74% of the radioactive dose detected in bile, urine and faeces, respectively; no radioactivity was detectable in the cage wash, GIT and its contents, or the residual carcass. It was stated that approximately 22% of the radioactivity in bile was reabsorbed via the GIT.

In Experiment 5, the main metabolite detected in plasma was Advantame-acid (~44/58 and 39/27% of plasma radioactivity in males/females at 0.5 and 2 h, respectively) followed by an unknown metabolite designated R4 (~26/17 and 0/10% of plasma radioactivity in males/females at 0.5 and 2 h, respectively). No parent compound was detected The minor metabolites, R1, R2 and R3, were detectable in both sexes at 0.5 h after dosing (~6/2, 7/5 and 9/16% of plasma radioactivity, respectively, in males/females), with R1 (both sexes), R2 (females) and R3 (males) increasing to 2 h after dosing (~24/16, 5/12 and 14/15% of plasma radioactivity, respectively, in males/females). Other undefined metabolites accounted for 2-8% of plasma radioactivity. No polar compounds were detected.

Conclusion

The GIT absorption of [^{14}C]-Advantame was approximately 11% based on the levels of radioactivity detected in urine (5-6%) and bile (5-6%) following a single gavage dose of 5 mg/kg bw. The majority (>90%) of radioactivity was excreted via the faeces. The predominant metabolites in urine and faeces were Advantame-acid and its demethylated derivative, RF-1, with no parent compound detectable. Up to four minor metabolites (<4% of the administered dose) were detected in bile, urine and faeces.

Aikens PJ, Kirkpatrick D, Kane TJ, Swan GJ & Milmoe S (2005e) ANS9801. Metabolism in the rat. Report No. AJO 194/042944. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. **GLP**: UK, EC, OECD, Japan & US FDA. **QA statement**: Yes.

Experimental

[^{14}C]-Advantame (>98% radiochemical purity; Batch No. AJO/139/TW1; synthesised by the performing laboratory) was mixed with unlabelled Advantame (99.9% purity; Batch No. 000825; sourced from the Sponsor) and administered to Han Wistar (Hsd Brl Han:WIST) rats as a gavage dose of 5 or 150 mg/kg bw in 1% (w/v) CMC (4 rats/sex/dose). Another group of 4 rats/sex were administered a single IV dose of 5 mg/kg bw [^{14}C]-Advantame, which had been mixed with [$^{13}C_6$]-Advantame (100% purity; Batch No. LFE/13C-ANS/82; synthesised by the performing laboratory) and unlabelled Advantame, in isotonic saline. The three dosing regimens were conducted as separate experiments. Rats were sourced from Harlan UK Ltd (Bicester, England), weighed 183-223 g and were a similar unspecified age. Urine and faeces were collected at 0-6, 6-12, 12-24, 24-48, 48-72 and 72-96 h after dosing. Cage washes were collected at 24 h intervals. Rats were sacrificed at 96 h after dosing; the GIT and its contents, and the residual carcass were retained for analysis. The concentration of radioactivity in samples was analysed by LSC, while urinary and faecal metabolites were analysed by HPLC with radiodetection.

Results

Mean levels of recovered radioactivity are summarised in Table 3.4. Total recovery was >97% within 96 h, with the majority detected in faeces (>96% and 72% following PO and IV dosing, respectively). Following IV dosing, excretion of radioactivity was predominantly via the faeces, with lower levels excreted via the urine (~75% *versus* 25% of the administered dose, respectively). Although the concentration of radioactivity in bile was not measured, based on the concentration of radioactivity in urine (<2%) and the observation that approximately three times as much radioactivity was detected in faeces following IV dosing, it is estimated that oral bioavailability was 8%. This estimate is in agreement with the direct determination of oral bioavailability of 7-9% (see Aikens et al 2004a). For the majority of rats, the highest urinary radioactivity was detected at the first sampling interval (0-6 h), while the highest faecal radioactivity was detected at 12-24 h. Most radioactivity had been excreted by 24 h, with excretion ostensibly complete by 48 h. In the majority of rats, no radioactivity was detectable in the GIT and its contents, or the residual carcass. Excretion patterns in males and females were similar.

TABLE 3.4: LEVELS OF RADIOACTIVITY RECOVERED IN URINE & FAECES*

Sample	Dose					
	5 mg/kg bw IV		5 mg/kg bw PO		150 mg/kg bw PO	
	Male	Female	Male	Female	Male	Female
Total	98.60±0.79	97.45±3.37	98.56±0.68	97.95±0.42	100.61±4.34	101.45±0.49

Sample	Dose					
	5 mg/kg bw IV		5 mg/kg bw PO		150 mg/kg bw PO	
	Male	Female	Male	Female	Male	Female
recovery						
Total Urine	25.84±2.91	23.90±9.36	1.67±0.14	1.94±0.44	0.97±0.07	1.49±0.82
Total Faeces	72.27±3.34	72.70±6.49	96.78±0.62	95.92±0.76	99.52±4.23	98.11±4.30
Total cage wash	0.41±0.30	0.85±0.71	0.12±0.03	0.09±0.02	0.12±0.07	1.85±3.38

*Results are expressed as the mean % of the administered radioactive dose ± 1 SD

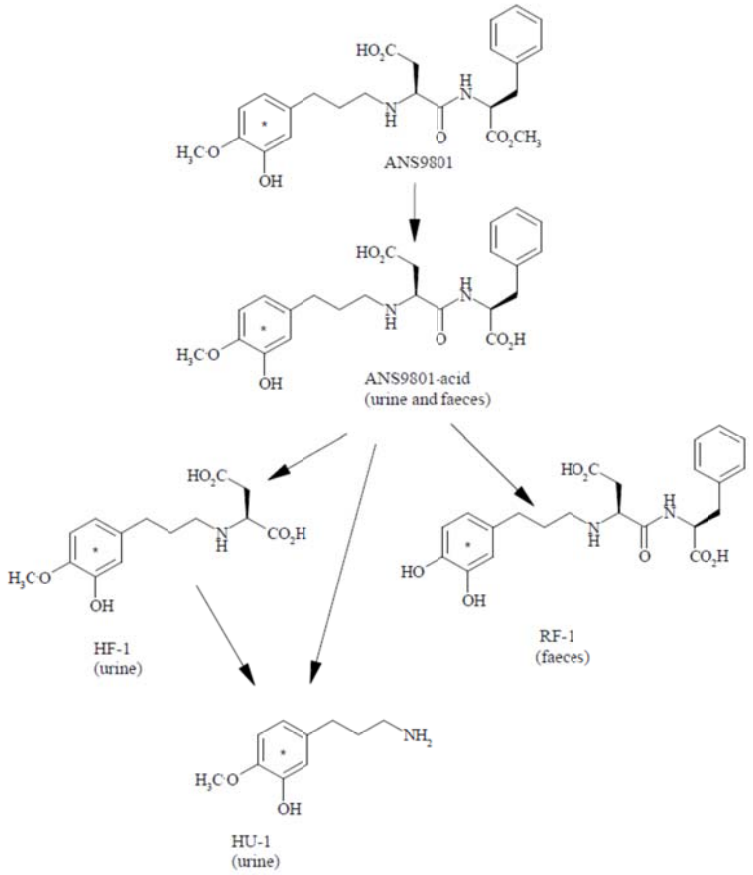
Metabolite profiles in pooled urine (0-24 h) and faeces (6-48 h) are summarised in Table 3.5. Overall, metabolites were qualitatively similar across all treatments. No unchanged Advantame was detected in urine or faeces following oral dosing; it was only detected in females following IV dosing. The main metabolite in urine following IV dosing was Advantame-acid (~98/93% of total urinary radioactivity in males/females). In contrast, Advantame acid constituted a smaller proportion of urinary radioactivity following oral dosing (~20-30% of total urinary radioactivity at the low dose and ~50% at the high dose) indicating extensive metabolism of absorbed Advantame-acid. Several of the metabolites were reported to be identical to those detected in human urine or faeces {HF-1 (N-(3-(3-hydroxy-4-methoxyphenyl))propyl-L-aspartic acid) from urine; HU-1 (3-[3-hydroxy-4-methoxyophenyl]-1-propylamine) from faeces}. At the low oral dose and IV dose, three faecal metabolites were identified (RF-2, RF1 and Advantame-acid), while only Advantame-acid was detected at the high oral dose.

TABLE 3.5: METABOLITES IDENTIFIED IN URINE & FAECES

Metabolite	Dose					
	5 mg/kg bw IV		5 mg/kg bw PO		150 mg/kg bw PO	
	Male	Female	Male	Female	Male	Female
Urine						
RU-1	nc	nc	<0.1	<0.1	<0.1	nc
HU-1	<0.1	<0.1	0.3	0.3	0.1	0.3
HF-1	0.1	0.4	0.3	0.3	0.1	0.1
RU-2	0.1	0.4	0.3	0.3	0.1	0.2
Z-1*	0.2	0.5	0.2	0.2	0.1	0.1
RU-3*	0.1	0.2	0.1	0.2	<0.1	<0.1
Advantame-acid	22.6	19.8	0.3	0.5	0.3	0.6
Advantame	nc	1.5	nd	nd	nd	nd
Others	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Faeces						
RF-2	8.1	8.3	11.5	11.9	nd	nd
RF-1	26.9	27.6	40.9	41.1	nd	nd
Advantame-acid	20.7	26.2	29.1	28.8	86.2	88.1
Others	9.4	6.5	8.8	7.4	7.3	5.5

Results are expressed as the % of the administered radioactive dose; nc = not calculable; nd = not detected;
*indistinct regions of radioactivity on the HPLC chromatogram containing at least 1 (Z-1) or 2 (RU3) radioactive components

Based on the results of these experiments, the authors proposed the following metabolic pathway for Advantame in rats:



Conclusions

Oral bioavailability was estimated to be 8%, with the main excretion pathway via the faeces. Advantame-acid was the main urinary metabolite, with up to 6 minor metabolites also identified. At the high oral dose, Advantame-acid was the only faecal metabolite identified, with two additional metabolites detected at the low oral dose and the IV dose. There was no difference in metabolism between males and females.

3.2.1.2 Dogs

***** Aikens PJ, Kirkpatrick D, Kane TJ & Holding (2005a) ANS9801. Metabolism in the dog. Preliminary investigations. Report No. AJO 180/013772. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. **GLP**: UK, EC, OECD, Japan & US FDA. **QA statement**: Yes.

[^{14}C]-Advantame (>98.0% radiochemical purity; Batch No. LFE/14C-ANS/65; synthesised by the performing laboratory) was mixed with unlabelled Advantame (99.9% purity; Lot No. 000825; sourced from the Sponsor) and administered to fasted beagle dogs (1/sex) as a single gavage dose of 5 mg/kg bw in 1% (w/v) CMC or as a single IV dose of 5 mg/kg bw in isotonic saline. Dogs were sourced from Harlan Interfauna UK Ltd and were six months old at dosing. Following dosing, urine and faeces were collected at 0-2 (IV group only), 0-6, 2-6 (IV group only), 6-12, 12-24, 24-48 and 48-72 h. Blood was collected at 0.25, 0.5, 1, 2, 4, 6, 8, 12 and 24 h (both groups), with additional blood samples taken at 0, 5, 10 and 45 min, and 48 and 72 h after IV dosing. Cage washes were collected every 24 h. Radioactivity was measured by LSC. Metabolites were analysed in pooled urine, faeces and plasma by HPLC with radiodetection. Based on the analysis of radioactivity in plasma, the following pharmacokinetic parameters were recorded or calculated: C_{max}, T_{max}, AUC_t, AUC, C_{last}, λ_z,

$T_{1/2}$, CL, V_z, and F.

Quantifiable concentrations of Advantame and Advantame-acid (and a metabolite designated D3) were detectable in plasma after the single oral or IV dose in both sexes. Except for immediately after the administration of the IV dose (when Advantame was the main analyte), Advantame-acid was the main analyte in plasma at all times over the 24 h (oral) or 72 h (IV) sampling periods. The apparently low levels of Advantame detected in plasma from 2 h after the single IV dose, or at all time-points following the single oral dose, were considered by the study authors to be an artefact of the analytical method.

Pharmacokinetic parameters derived from plasma concentrations of radioactivity are summarised in Table 3.6. Following the single oral dose, radioactivity was absorbed and eliminated relative slowly, with T_{max} and estimated $T_{1/2}$ values of 6 and 70 h, respectively. The bioavailability was 6% in males and 14% in females, which was also reflected in the higher C_{max} and AUC values in females.

TABLE 3.6: PHARMACOKINETIC PARAMETERS FOR DOGS

Parameter	5 mg/kg bw, IV		5 mg/kg, PO	
	Male	Female	Male	Female
C_{max} (µg eq/g)	-	-	0.75	1.68
T_{max} (h)	-	-	6	6
AUC_{24} (µg eq.h/g)	252.58	255.68	15.70	34.30
AUC_{72} (µg eq.h/g)	-	-	40.87	90.69
λ_z (h^{-1})	0.0151[a]	0.0212[a]	0.0099[a]	0.0099[a]
$T_{1/2}$ (h)	46.0[a]	32.7[a]	70.0[a]	70.0[a]
F (%)	-	-	6%	14%

a = estimate only as the it did not meet the following criteria: (i) The terminal data points were apparently randomly distributed about a single straight line (on visual inspection); (ii) A minimum of 3 data points was available for the regression; (iii) The regression coefficient was >0.95, and the fraction of the variance accounted for was >0.90; (iv) The interval including the data points chosen for the regression was at least two-fold greater than the half-life itself.

Recovery of radioactivity was 97.44/95.83% of the administered dose (male/female), with most detected in faeces (92.51/85.73%) and relatively low levels detected in urine (3.91/7.48%). The highest radioactivity was detected in the 0-6 h urine sample and 6-12 in faecal sample. The majority of the administered dose was eliminated by 24 h. Following a single IV dose, recovery of radioactivity was 73.19/77.68% (male/female), with urine and faeces containing 33.77/33.15 and 38.07/43.17% of the administered dose, respectively. Peak urinary radioactivity following IV dosing was detected in the 6-12 h sample in the male and 2-6 h in the female, while in faeces it was 12-24 h for both sexes. The lower level of recovered radioactivity in dogs following IV dosing compared to oral dosing was attributable to the higher retention of radioactivity. Based on the level of the urinary radioactivity following oral and IV dosing, the level of GIT absorption was estimated to be 11.6% in males and 22.6% females. However, these estimates are not considered accurate in light of the relatively slow elimination of radioactivity from plasma and concomitant short duration of urine sampling. It is likely that the short sampling duration is the main reason for the investigators repeating the study with an extended sampling interval (see below).

With the exception of samples collected immediately at the end of the IV infusion where Advantame was the main analyte, Advantame-acid was the main metabolite in plasma, urine and faeces. Three additional minor metabolites, designated D1, D2 and D3, were detected in urine, with D1 and D3 also detected in faeces and plasma, respectively.

Aikens PJ, Kirkpatrick D, Kane TJ, Swan GJ, Milmoe S, Baldrey SF & McBurney (2005b) ANS9801. Metabolism and pharmacokinetics in the dog. Report No. AJO 193/042943. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. **GLP**: UK, EC, OECD, Japan & US FDA. **QA statement**: Yes.

Experimental

[^{14}C]-Advantame (>98% radiochemical purity; Batch No. AJO/139/TW1; synthesised by the performing laboratory) mixed with unlabelled Advantame (99.9% purity; Batch No. 000825; sourced from the Sponsor) was administered sequentially to 3 beagle dogs/sex as a single gavage dose of 5 mg/kg bw, a single gavage dose of 150 mg/kg bw (both in 1% w/v carboxymethyl cellulose) or a single IV dose of 5 mg/kg bw (in isotonic saline). Dogs were subjected to at least a 4-week washout period between treatments to ensure that there was no detectable radioactivity in plasma and urine. Dogs were sourced from Harlan USA, weighed 9.0-11.3 kg and were approximately 6 months old at the time of first dosing. Dogs were fasted prior to dosing, with the study report stating that food and water were available *ad libitum* from 4 h after dosing.

Blood samples were collected immediately prior to, and then at 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72, 96 and 120 h after, dosing. Urine and faeces were collected separately at 0-6, 6-12, 12-24, 24-48, 48-72, 72-96 and 96-120 h after dosing. Following IV dosing, blood samples were collected immediately prior to and after infusion, and then at 5, 10, 15 and 30 min and 1, 2, 6, 12, 24, 48, 72, 96, 120, 144 and 160 h after dosing. Urine and faeces were collected separately at 0-2, 2-6, 6-12, 12-24 and every 24 h thereafter to 168 h. Cage washes were collected at 24 h intervals. Radioactivity was analysed by LSC, while the concentrations of Advantame and Advantame-acid were measured by LC-MS/MS. Radioactive metabolites were analysed by HPLC with radiodetection. The following pharmacokinetic parameters were recorded or calculated: C_{max}, T_{max}, AUC_t, AUC, C_{last}, λ_z, $T_{1/2}$, CL, V_{ss}, V_z, and F.

Results

PHARMACOKINETICS

With the exception of the sample collected immediately after the administration of the IV dose (when Advantame was the main analyte), Advantame-acid was the main analyte in plasma. At the low oral dose, only one dog (male) had quantifiable plasma concentrations of Advantame from 0.5-4 h after dosing, while Advantame-acid was quantifiable to 48 h after dosing. At the high oral dose, Advantame and Advantame-acid were quantifiable in all dogs from 0.5-2 h and 0.5-120 h after dosing, respectively. Following the IV dose, Advantame was quantifiable in plasma from all dogs from 0-2 h after dosing, with Advantame-acid quantifiable from 0.08-168 h after dosing. Approximately 50% of the administered dose of Advantame had been converted to Advantame-acid by 10 min, with conversion completed by 6 h.

Pharmacokinetic parameters for total plasma radioactivity and Advantame-acid are summarised in Table 3.7. Following oral dosing, GIT absorption of radioactivity was relatively slow, with T_{max} values of 7 or 8 h at 5 mg/kg bw and 6 h at 150 mg/kg bw. This contrasts with a T_{max} of ~1 h for Advantame-acid. There was a dose-related increase in mean C_{max} and AUC values for radioactivity following oral dosing, which was less-than proportional to the dose increment (17 to 24-fold *versus* the expected 30-fold increase for linear kinetics). An examination of C_{max} and AUC_t values for Advantame-acid indicated that the increase from the low to high oral dose was also less than the dose increment. Collectively, these findings suggest non-linear kinetics over the tested dose range for Advantame-acid and its metabolites.

A comparison of AUC_t values for Advantame-acid with those for total radioactivity indicate that Advantame-acid accounted for approximately 0.5% of absorbed components at the low and high oral doses. These findings are difficult to reconcile with the HPLC analysis of radioactive components in urine (see below), where Advantame-acid accounted for approximately 50% of total urinary radioactivity at both the low and high oral doses. This discrepancy suggests a problem with the new analytical method used in this study but might also be a product of the use of two different analytical methods to measure Advantame-acid. However, the findings of both methods (LC-MS/MS *versus* HPLC with radiodetection) indicate that absorbed Advantame-acid is extensively metabolised.

The $t_{1/2}$ of plasma radioactivity following oral or IV dosing was 74-95 h; the authors attributed this relatively long half-life to the slow elimination of sulfate conjugates of Advantame-acid, which they stated accounted for ~99% of the recovered radioactivity in plasma[8]. As sulfation is known to increase the water solubility of a compound enabling it to be excreted more readily via the urine or bile, it is difficult to reconcile the argument provided by the authors with the absence of any sulfate conjugates of Advantame-acid in the urine or faeces (see below). A more likely explanation for the relatively long elimination half-life of radioactivity in dogs is enterohepatic re-circulation. However, this is unlikely to be due to the recirculation of Advantame-acid, which is extensively metabolised following oral absorption and given that the apparent $t_{1/2}$ for Advantame-acid was 4.2-7.1 h. The bioavailability of radioactivity was approximately 14-15% and 8-9% at the low and high oral doses, respectively.

TABLE 3.7: PHARMACOKINETIC PARAMETERS FOR DOGS

Parameter	5 mg/kg bw, IV		5 mg/kg bw, PO		150 mg/kg bw, PO	
	Males	Females	Males	Females	Males	Females
Radioactivity[a]						
C_{max} (µg eq./g)	-	-	1.47	1.85	34.99	32.90
T_{max} (h)	-	-	7	8	6	6
AUC_t (µg eq.h/g)	942	1058	108.1	138.6	2518	2423
AUC_∞ (µg eq.h/g)	1266	1498	175.5	220.6	3730	3842
K_{el} (h^{-1})	0.008	0.0073	0.0081	0.0086	0.0094	0.0086
$T_{1/2}$ (h)	86.3	95.0	85.6	80.6	74.0	80.9
F (%)	-	-	14.9	14.3	9.4	8.4
Advantame-acid						
C_{max} (µg/g)	-	-	0.186	0.212	3.320	4.070
T_{max} (h)	-	-	0.5	0.5	0.5	1
AUC_t (µg.h/g)	1.7	1.5	0.585	0.737	9.95	11.1
AUC_∞ (µg.h/g)	1.92	1.6	0.698	0.843	12.0	13.4
K_{el} (h^{-1})	1.15	1.38	0.15	0.16	0.097	0.13
$T_{1/2}$ (h)	0.6	0.5	4.7	4.2	7.1	5.5

Results expressed as means; a = concentrations expressed as Advantame equivalents

EXCRETION

The level of radioactivity in excreta is summarised in Table 3.8. The lower recovery observed following IV dosing is suggestive of an inadequate sampling duration. Excreta were collected for only 168 h. For a compound with an apparent terminal half-life in plasma of around 83-90 h this duration is unlikely to be adequate. By comparison the apparent terminal half-life in rat plasma is around 8 h and excreta were collected for 96 h (Table 3.1). A probable consequence of this short sampling duration relative to the apparent half-life is that the ratio

[8] The authors stated that the new analytical method used in this study was capable of distinguishing sulfate conjugates of Advantame-acid from Advantame-acid, which accounted for the majority of the recovered radioactivity in plasma. However, no data were provided to confirm the presence of sulfate conjugates in plasma.

of excreted radioactivity in urine to faeces following IV dosing may be a somewhat less accurate estimate relative to the rat where the recoveries were essentially complete (Table 5).

Following IV dosing, almost equal proportions of radioactivity were detected in faeces and urine, with the highest levels detected during the first 6 h and from 2-24 h, respectively. The detection of radioactivity in faeces following IV dosing indicates excretion via the bile and/or GIT secretion. Following oral dosing, the majority of radioactivity was detected in faeces, with relatively low levels detected in urine. However, for one male in the low dose group, more radioactivity was detected in urine (~44%) than faeces (~34%); this result is considered anomalous. The highest urinary and faecal radioactivity was detected in the 0-6 h and 6-12 or 12-24 h samples, respectively, with the majority excreted by 24 h. Low levels of radioactivity were still detectable in faeces at the last sampling point following oral (96-120 h) or IV dosing (144-168 h). Based on the level of radioactivity in urine following oral dosing (4-7% of the administered radioactive dose) and the observation that approximately half the radioactivity was detected in faeces following IV dosing, bioavailability following oral administration is estimated to be 8-14%. This estimate is in good agreement with the F-value determined from pharmacokinetic data.

TABLE 3.8: LEVELS OF RADIOACTIVITY IN POOLED EXCRETA

Sample	5 mg/kg bw, IV		5 mg/kg bw, PO		150 mg/kg bw, IV	
	Males	Females	Males	Females	Males	Females
Total recovery	84.0	85.9	92.8	94.3	94.6	94.9
Urine	39.1	37.1	7.3	7.4	5.2	3.7
Cage wash	3.0	1.4	3.2	1.8	2.5	2.2
Faeces	41.9	47.4	82.4	85.0	87.0	89.0

Results expressed as the mean % of the administered radioactive dose

METABOLITE ANALYSIS

Specific analysis for plasma metabolites was not undertaken. Advantame and Advantame-acid were quantifiable in plasma; the concentration of Advantame-acid was higher than that of Advantame. The results of the analysis of metabolites in pooled urine are summarised in Table 3.9. The main radioactive metabolite in urine following IV dosing was Advantame-acid (~70% of total urinary radioactivity). In contrast, Advantame-acid constituted ~50% of total urinary radioactivity at both the low and high oral doses indicating extensive metabolism of absorbed Advantame-acid. Advantame was only detected in urine following IV dosing. At the low oral dose, the metabolite designated HF-1 was present at a relatively high level (~3 times less than Advantame-acid), while the metabolite, D3, was the second highest metabolite following IV dosing. The authors stated that no sulfate conjugates of Advantame-acid were detected in urine.

TABLE 3.9: LEVELS OF URINARY METABOLITES

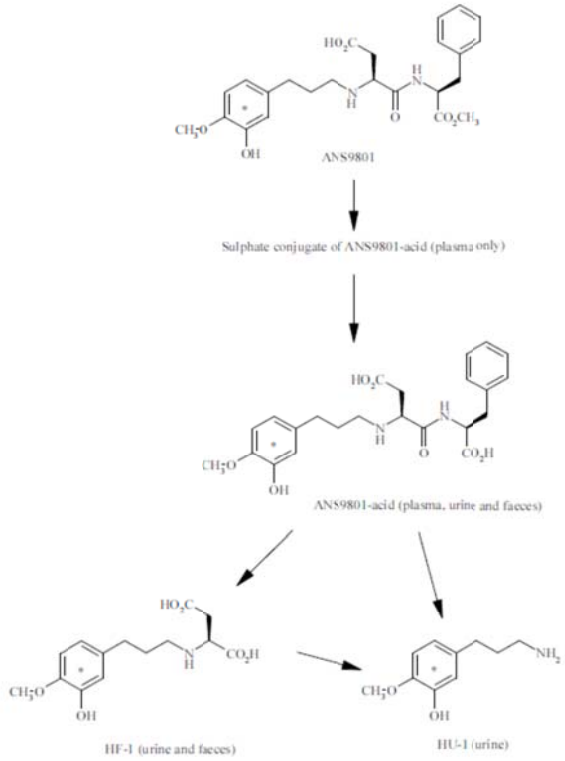
Metabolite	Dose					
	5 mg/kg bw, IV		5 mg/kg bw PO		150 mg/kg bw PO	
	Male	Female	Male	Female	Male	Female
HU-1	0.2	0.3	0.3	0.3	0.2	0
HF-1	0.9	1.4	1.3	1.5	0.3	0.1
ZD-1	0.1	0.1	0.3	0.2	0.1	0
ZD-2	0.2	0.2	0.2	0.1	0.1	0
D3	5.1	4.5	1.0	0.9	0.8	0.1
AA	25.1	22.9	3.0	3.1	2.4	0.5
A	2.8	3.4	<0.1	<0.1	<0.1	<0.1
others	0.5	0.5	0.1	0.1	0.1	0.1

Results are expressed as the mean % of the administered radioactive dose; HU = 3-[hydroxyl-4-methoxyphenyl]-

1-propylamine; HF-1 = N-(3-(3-hydroxy-4-methoxyphenyl) propyl-L-aspartic acid; AA = Advantame-acid; A = Advantame

Two metabolites were identified in faeces at 6-12 h, with their concentrations declining (to undetectable levels in some cases) in the 12-24 and 24-48 h pooled samples. Advantame-acid was the main metabolite in all cases (41.4/62.7.3%, 58.4/71.3% and 16.6/14.5% in males/females following the low and high oral dose, and IV dose, respectively, at 6-12 h). HF-1 was a minor metabolite in faeces but in total accounted for no more than approximately 4%, 1% and 3% of the administered dose following the low and high oral dose, and IV dose, respectively. Two regions of the HPLC chromatogram containing unresolved radioactivity, designated Zone 1 and Zone 2, were also described by the study authors, but in total accounted for no more than 0.7 and 2.1%, 0.4 and 1.6%, and 0.6 and 2.6%, respectively, of the administered low and high oral dose, and IV dose, respectively. Other unidentified metabolites accounted for less than ≤0.8% of the administered radioactive dose. The authors stated that no sulphate conjugates of Advantame-acid were detected in dog faeces.

Based on the results of these experiments, the authors proposed the following metabolic pathway for Advantame in dogs:



As discussed above, FSANZ considers that insufficient evidence has been provided to substantiate the presence of a sulphate conjugate of Advantame-acid.

Aikens PJ, Kirkpatrick D, Pluthero MG & Kane TJ (2002b) ANS9801. Tissue distribution in the male dog. Report No. AJO 191/022818. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. **GLP**: UK, EC, OECD, Japan & US FDA. **QA statement**: Yes.

[^{14}C]-Advantame (98.6% radiochemical purity; Batch No. AJO139/TWI; synthesised by the performing lab) was mixed with unlabelled Advantame (99.9% purity; Lot No. 000825; sourced from the Sponsor) and administered to four beagle dogs as a gavage dose of 5 mg/kg bw in 1% (w/v) CMC. Dogs were obtained from Harlan Hillcrest UK Ltd, weighed 9.0-

10.0 kg and were approximately 5-7 months old at dosing. One dog was sacrificed at 6, 72, 144 and 288 h after dosing. With the exception of the first dog sacrificed, blood was collected immediately prior to dosing and at 1, 2, 4, 6, 8, 12, 24 and every 24 h thereafter until sacrifice. Various organs/tissues were sampled following sacrifice. Radioactivity was analysed in blood and tissue samples by LSC.

The highest radioactivity was detected in the dog sacrificed at 6 h after dosing, with decreasing concentrations measured in subsequent dogs. Radioactivity was detectable in all sampled tissues, with the highest concentrations measured in the large intestine wall, bile, plasma, liver, bladder and kidney (69.21, 10.58, 0.805, 0.629, 0.325 and 0.313 µg equivalent Advantame/g, respectively, in the dog sacrificed 6 h after dosing). Only the bile and large intestine wall had a tissue:plasma ratio >1. The tissue:plasma ratio was relatively constant over time suggesting that the concentration of tissue radioactivity was a due to the blood flow to that tissue. There was no evidence of accumulation in any tissue/organ.
Peak plasma radioactivity occurred at 6 (2 dogs) or 24 h (1 dog) after dosing, while the mean half-life was 71 h (range 56-95 h). The mean K_{el} was 35 sec^{-1} (range 26-45 sec^{-1}). The rate of decline in tissue radioactivity was similar to that of plasma.

Aikens P, Kane TJ & Milmoe S (2005f) ^{14}C-ANS9801. Comparison of metabolite profiles in rats, dogs and humans. Report No. AJO 218/042955. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. **GLP**: Yes (UK, EC, OECD, Japan & US FDA). **QA statement**: Yes.

This study compared the metabolite profiles in urine and faeces from humans, rats and dogs dosed orally with radiolabelled Advantame. Excreta that had been collected and stored frozen at -15°C during previous experiments (AJO 210/042812, AJO 194/042944 & AJO 193/042943) were re-analysed by TLC and HPLC (with radiodetection). Three metabolites were detected in human urine (Advantame-acid, HU-1 and HF-1) and these were consistent with those identified in dogs and rats. In all three species, Advantame-acid was the main metabolite in faeces. Additional faecal metabolites included HF-1 (humans and dogs) and RF-1 (rats only). Treatment of dog urine with glucuronidase/sulfatase generated no new compounds indicating the absence of glucuronidated or sulfated conjugates.

3.2.2 *In Vitro* Studies

Baldrey SF, Flack I, Phelan M, Williams M & Boussalt A (2002a) ANS9801 and ANS9801-Acid. Validation of a LC-MS/MS bioanalytical method for the measurement of ANS9801 and ANS9801-Acid in rat plasma. Report No. AJO 147/012240. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. **GLP**: UK, EC, OECD, Japan & US FDA. **QA statement**: Yes.

Baldrey SF, Flack I, Phelan M, Williams M & Boussalt A (2002b) ANS9801 and ANS9801-Acid. Partial validation of a LC-MS/MS bioanalytical method for the measurement of ANS9801 and ANS9801-Acid in dog plasma. Report No. AJO 153/012241. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. **GLP**: UK, EC, OECD, Japan & US FDA. **QA statement**: Yes.

Baldrey SF, Flack I & Phelan M (2002c) ANS9801 and ANS9801-Acid. Partial validation of a LC-MS/MS bioanalytical method for the measurement of ANS9801 and ANS9801-Acid in mouse plasma. Report No. AJO 169/014336. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. **GLP**: UK, EC, OECD, Japan & US FDA. **QA statement**: Yes.

Baldrey SF, Flack I & Phelan M (2002d) ANS9801 and ANS9801-Acid. Partial validation of a LC-MS/MS bioanalytical method for the measurement of ANS9801 and ANS9801-Acid in rabbit plasma. Report No. AJO 170/014335. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. **GLP**: UK, EC, OECD, Japan & US FDA. **QA statement**: Yes.

Bates T, Baldrey S, Shafait S & Low K (2005) ANS9801 and ANS9801-Acid. Validation of a LC-MS/MS bioanalytical method for the measurement of ANS9801 and ANS9801-Acid in human plasma. Report No. AJO 206/032893. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. **GLP**: UK, EC, OECD, Japan & US FDA. **QA statement**: Yes.

A series of studies were undertaken to develop validated or partially validated LC-MS/MS methods to analyse Advantame and Advantame-acid in rat, dog, mouse, rabbit and human plasma. Advantame and Advantame-acid were added to plasma that had been stabilised with citric acid and/or the esterase inhibitor, paraoxon (with the exception of human plasma), at target concentrations of 1.5 and 70 ng/mL (rat, dog, rabbit), 3.0 and 140 ng/mL (mouse) or 1.5 and 3 ng/mL (human). In animal plasma, Advantame and Advantame-acid were stable for 2 h at room temperature, 3-7 months at -80°C and following 2-3 freeze/thaw cycles. Advantame and Advantame-acid were stable in human plasma for ~4 h at room temperature, for up to 12 months at -80°C and following 3 freeze/thaw cycles. Following extraction from human plasma, both compounds were stable for at least 24 h at room temperature and 20 days at 4°C.

Aikens PJ, Kirkpatrick D, Pluthero MG & Wallbank K (2002c) ^{14}C-ANS9801 and ^{14}C-ANS9801-acid: Stability in simulated gastric and intestinal fluid. Report No. AJO 173/013290. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. **GLP**: UK, EC, OECD, Japan & US FDA. **QA statement**: Yes.

[^{14}C]-Advantame (98.7% radiochemical purity; Batch No. LFE/14C-ANS/65; synthesised by the performing laboratory) or [^{14}C]-Advantame-acid (98.6% radiochemical purity; Batch No. LFE/14C-ANSACID/3; synthesised by the performing lab), in a mixture with the corresponding unlabelled compound, were incubated in simulated gastric fluid (SGF) or simulated intestinal fluid (SIF) in the presence and absence of pepsin or pancreatin for up to 2 h at 37ºC. A single concentration of each compound was tested (50 µg/mL Advantame and 25 µg/mL Advantame-acid). SGF and SIF were prepared according to the US Pharmacopoeia (1995). The concentrations of Advantame and Advantame-acid were

analysed using HPLC, with radiodetection, in samples collected at 0, 1, 5, 15, 30, 60 and 120 min.

Advantame was relatively stable in SGF, with low levels of Advantame-acid detectable only at 120 min (3.5% of total radioactivity). In the absence of pepsin, no Advantame-acid was generated. Advantame-acid was completely stable in SGF in the presence and absence of pepsin. In SIF, Advantame was completely hydrolysed to Advantame-acid within 5 min, with only limited hydrolysis occurring in the absence of pancreatin (3.4% of total radioactivity detected as Advantame-acid at 120 min). Advantame-acid was relatively stable in SIF, with a loss of only 1.8% detected at 120 min; there was no loss of Advantame-acid in the absence of pancreatin.

Aikens PJ & Hickson JA (2004) ^{14}C-ANS9801 and ^{14}C-ANS9801-acid. Studies of plasma protein binding *in vitro* (rat, dog and human). Report No. AJO 213/033887. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. **GLP**: UK, EC, OECD, Japan & US FDA. **QA statement**: Yes.

The *in vitro* binding of [^{14}C-Advantame] or [^{14}C]-Advantame-acid (99.1% purity; Batch No.s COLTW/4 & TW/F25-27, respectively; both compounds synthesised by the performing laboratory) to proteins in pooled rat, dog and human plasma was analysed. Various concentrations of both compounds were incubated with plasma for 10 min at 37°C; protein-bound ligand was separated from unbound ligand by centrifugal ultrafiltration and radioactivity measured by LSC. The results are summarised in Table 3.10 and indicate that both compounds readily bound to plasma proteins and that binding was not saturable over the tested concentration ranges.

TABLE 3.10: RESULTS OF PLASMA PROTEIN BINDING

Plasma sample	Total protein (g/L)	Total albumin (g/L)	Conc. Advantame & Advantame-acid (ng/mL)	Binding
Rat 15 male; Han-Wistar (Hsd Brl Han: WIST)	58	35	10, 100, 1000 & 10000 (Advantame-acid only)	90.5-91.8%
Dog 17 male beagle	53	31	20, 200, 2000 & 20000	Advantame: 63.1-64.9% Advantame-acid: 61.-71.3%
Human 3 healthy males	74	45	10, 100, 500 & 1000	Advantame: 81.3-92.4% Advantame-acid: 96.2-96.9%

3.2.3 Acute Toxicity Studies

3.2.3.1 Rats

Blanchard EL & Clemson AD (2001) ANS9801: Acute oral toxicity to the rat (acute toxic class method). Report No. AJO 155/012600/AC. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. **GLP**: UK, EC, OECD, US FDA & Japan. **QA statement**: Yes. **Test Guidelines** EEC, OECD & US FDA

Advantame (99.9% purity; Lot No. 000825; sourced from the Sponsor) was administered as a single gavage dose to 5 fasted female Wistar (Han) rats (5-7 weeks of age; 85-94 g bodyweight; sourced from Harlan UK Ltd, Oxon, England) at a dose of 5000 mg/kg bw in 1% (w/v) aqueous methyl cellulose. Rats were housed together, with food and water available *ad libitum* from 4 h after dosing. Observations for mortalities and clinical signs were made twice daily. Bodyweight was recorded the day before dosing, the day of dosing and at d 7 and 14. Survivors were sacrificed 14 days after dosing and necropsied.

There were no mortalities. One rat exhibited increased salivation immediately after dosing, while all rats had white coloured faeces on d 2; all rats appeared normal by d 3. Bodyweight gains were unremarkable and there were no treatment-related macroscopic abnormalities. To confirm these observations, 5 male rats were similarly dosed; the only treatment-related abnormality was the occurrence of white-coloured faeces. The LD_{50} in rats was therefore >5000 mg/kg bw.

Williams CN & Murphy AM (2001) ANS9801: Irwin dose-range in rats following oral administration. Report No. AJO 161/012397. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, AminoScience Division, Tokyo, Japan. **GLP**: UK, EC, OECD, US FDA & Japan. **QA statement**: Yes.

Advantame (99.9% purity; Lot No. 000825; sourced from the Sponsor) was administered as a single gavage dose to 6 male Wistar (Han) rats per group at doses of 0, 10, 100 or 1000 mg/kg bw in 1% (w/v) aqueous methyl cellulose. The dose volume was 4 mL/kg bw. Rats were sourced from Charles River (UK) Ltd, weighed 142-166 g and were approximately 7 weeks of age at dosing. They were housed 3/cage, with food and water available *ad libitum* except that they were fasted overnight prior to dosing and then until 180 min post-dose. Behaviour was assessed at 30, 60, 180 and 300 min, and 24 h after dosing, with daily observations for mortalities and clinical signs made until 7 days post-dose. Rats were sacrificed at the end of the 7-day observation period. No deaths or behavioural abnormalities were observed.

Williams CN & Adams AR (2001) ANS9801: Assessment of locomotor activity in rats following oral administration. Report No. AJO 162/012597. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, AminoScience Division, Tokyo, Japan. **GLP**: UK, EC, OECD, US FDA & Japan. **QA statement**: Yes.

Advantame (99.9% purity; Lot No. 000825; sourced from the Sponsor) was administered as a single gavage dose to 10 male Wistar (Han) rats per group at doses of 0, 10, 100 or 1000 mg/kg bw in 1% (w/v) aqueous methyl cellulose. The dose volume was 4 mL/kg bw. A positive control group of 10 rats was given a single gavage dose of 3 mg/kg bw amphetamine sulfate in 10% (w/v) methyl cellulose. Rats were sourced from Charles River (UK) Ltd, weighed 133-193 g and were approximately 6 weeks of age at dosing. They were housed 5/cage, with food and water available *ad libitum* except that they were fasted overnight prior to dosing. The spontaneous locomotor activity of each rat was recorded for 10 min, prior to dosing and at 30, 60, 180 and 300 min after dosing, using an automated activity monitor. Rats were sacrificed at the end of the 300 min observation period. The positive control gave the expected results [an increase in spontaneous locomotor activity relative to the vehicle control, which was statistically significant ($p<0.05$) at the 30, 60 and 180 min intervals]. There was no treatment-related effect on spontaneous locomotor activity based on the absence of a dose-response relationship and any statistical differences between the test and vehicle control group.

Williams CN, Murphy AM & Smith M (2001) ANS9801: Charcoal propulsion test in rats following oral administration. Report No. AJO 164/012575. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. **GLP**: UK, EC, OECD, US FDA & Japan. **QA statement**: Yes.

The effect of Advantame (99.9% purity; Lot No. 000825; sourced from the Sponsor) on gastrointestinal motility was assessed following the administration of a single gavage dose to 10 male Wistar (Han) rats per group at doses of 0, 10, 100 or 1000 mg/kg bw in 1% (w/v) aqueous methyl cellulose. The dose volume was 4 mL/kg bw. A positive control group of 10 rats was dosed with 10 mg/kg bw morphine sulfate. Rats were sourced from Charles River (UK) Ltd, weighed 139-169 g, were approximately 7 weeks of age and fasted overnight prior to dosing.

Thirty min after dosing, each rat was gavaged with 1.0 mL charcoal (5% w/v) in water and sacrificed 30 min later. The GIT was removed and the distance migrated by the charcoal from the pyloric sphincter measured (results were expressed as a percentage of the total gut length).

Gastrointestinal motility was unaffected by treatment at 10 and 100 mg/kg bw, while there was a significant decrease ($p<0.01$) at 1000 mg/kg bw relative to the control group (37.6±5.74 *versus* 52.6±11.08%, respectively). The authors attributed this result to the bulking effect of the high-dose formulation rather than to a specific pharmacological effect. Motility was significantly lower in the positive control group relative to the vehicle control group (30.4±10.33 *versus* 52.6±11.08%, respectively).

3.2.3.1 Dogs

Jordan SM, Williams CN, Davies C & Murphy A (2001) ANS9801: Cardiovascular and respiratory evaluations in the anaesthetised dog following intraduodenal administration. Report No. AJO 163/012426. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, AminoScience Division, Tokyo, Japan. **GLP**: UK, EC, OECD, US FDA & Japan. **QA statement**: Yes.

Advantame (99.9% purity; Lot No. 000825; sourced from the Sponsor) was administered as a single intraduodenal bolus dose to 3 anaesthetised beagle dogs per group at doses of 0, 10, 100 or 1000 mg/kg bw in 1% (w/v) aqueous methyl cellulose. The dose volume was 2 mL/kg bw. Dogs were sourced from Harlan Hillcrest (England), weighed 9.8-13.8 kg, approximately 7-12 months of age and fasted for a minimum of 16 h prior to dosing. The following cardiovascular and respiratory parameters were recorded at 5 min intervals during a 30 min stabilisation period (prior to dosing), at 10 min intervals for the first h after dosing and thereafter every 15 min up to 240 min: arterial blood pressure; heart rate; left ventricular systolic pressure and dp/dt max (cardiac contractility); ECG; femoral blood flow; femoral resistance; respiration rate, respiratory minute volume and tidal volume. Dogs were sacrificed at the end of the observation period.

The mean arterial blood pressure and tidal volume of the 100 mg/kg bw group was significantly lower and higher, respectively, than the control group (p values unspecified) but in the absence of any differences at the highest dose these findings were considered incidental in nature. There was no treatment-related effect on any of the measured cardiovascular or respiratory parameters.

3.2.4 Short-Term Repeat-Dose Toxicity Studies

3.2.4.1 Rats

Chase KC (2002a) ANS9801: Preliminary toxicity study by dietary administration to Han Wistar rats for 4 weeks. Report No. AJO 151/012004. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. **GLP**: UK, EC, OECD, US FDA & Japan. **QA statement**: Yes.

***** Baldrey SF, Phelan MR, McBurney & Holding J (2002e) Pharmacokinetics of ANS9801 and ANS9801 following a maximum tolerated dosage study by dietary administration to Han Wistar rats. Report No. AJO 151/012004. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. **GLP**: UK, EC, OECD, US FDA & Japan. **QA statement**: Yes.

Experimental

Advantame (99.2% purity; Batch No. 000530; sourced from the Sponsor) was admixed in the diet and fed *ad libitum* to groups of 10 Han Wistar rats/sex at concentrations of 0, 1500, 15000, 30000 or 50000 ppm for 4 weeks. Rats were sourced from Harlan UK Ltd (Oxforshire, England), weighed 80-116 g, were approximately 38-42 days old at the start of treatment and housed individually throughout the dosing period.

Observations for mortalities and clinical signs were made at least twice daily, with a more detailed examination performed on a weekly basis. Bodyweight was recorded prior to dosing, the day that dosing commenced and twice weekly thereafter. Food consumption was recorded weekly. Water consumption was recorded over 3 days during wk 1 and 3. Ophthalmoscopic examinations were performed on all rats prior to dosing and then on the control and high-dose groups during wk 4. The standard range of haematology, clinical chemistry and urinary parameters (Appendix 1) were analysed during wk 4 (with the exception of reticulocytes, LDH, CPK, urobilinogen and reducing substances). For toxicokinetic analysis, the concentrations of Advantame and Advantame-acid were measured in blood samples collected during wk 1 and 4; standard toxicokinetic parameters were recorded or calculated.

Following sacrifice on d 28 (males) or 29 (females), all rats were necropsied. The standard organs were weighed (Appendix 2) in addition to the lungs (with mainstem bronchi), salivary glands and thymus. Standard organs/tissues were preserved for histopathology (Appendix 2) (with the exception of the Harderian and Zymbal's glands) and examined for the control and high-dose groups. The kidneys of all treated females were histopathologically examined as was any rat exhibiting macroscopic tissue abnormalities.

Results

MORTALITIES & CLINICAL SIGNS

There were no deaths and no treatment-related clinical signs. At d 15, the incidence of light green staining of the cage lining was observed across all groups, including the control (60%, 73%, 94%, 78% and 94% at 0, 1500, 30000 and 50000 ppm, respectively), which the authors stated was associated with the urine. Over the remainder of the study, the incidence of this finding decreased. Given the occurrence of this finding in the control group, it is not considered treatment-related. For some treated rats, sporadic occurrences of dark green, light purple or blue staining of the cage lining were also noted, which were associated with contact of faeces with the lining.

The occurrence of discoloured faeces (striped, pale or dark) was also increased in treated

groups from d 15, tending to increase with dose and over the duration of treatment. These observations are not considered toxicologically significant and are likely due to the relatively high concentration of Advantame and/or its metabolites in faeces.

BODYWEIGHT & FOOD/WATER CONSUMPTION

At 50000 ppm, the mean bodyweight of males and females was significantly lower ($p<0.05$ or 0.01; 6-10% lower) than control from d 7-10. Bodyweight gain over 4-weeks was also significantly lower than the control [Males: 102±14.2 *versus* 125±10.1 g, respectively ($p<0.01$); Females: 50±9.0 *versus* 59±7.8 g, respectively ($p<0.05$)]. There was no effect on bodyweight or bodyweight gain in any of the other treatment groups. Food consumption was unaffected by treatment.

On a weekly basis, significantly reduced food conversion efficiency was determined at 30000 (wk 1 & 2; 11 & 15% lower than the control, respectively) and 50000 ppm (wk 1, 2 & 4; 25, 15 & 57% lower than the control, respectively) in males. In females, significantly reduced food conversion efficiency was determined only at 50000 ppm during wk 1 ($p<0.05$; 8% lower than the control). Overall (wk 1-4) food conversion efficiency was approximately 20% lower in males at 30000 and 50000 ppm, while in females, it was approximately 15% lower at 50000 ppm.

The apparent effect on bodyweight gain and food conversion efficiency is considered attributable to the high concentration of a non-caloric substance in the diet rather than to a direct toxicological effect.

Mean achieved doses of Advantame over four weeks were 174/187, 1739/1920, 3672/3739 and 6126/6490 mg/kg bw/day in males/females at 1500, 15000, 30000 or 50000 ppm, respectively.

The mean water consumption of males was significantly higher ($p<0.05$ or 0.01) than the control group at and above 1500 ppm during wk 1 (25±2.7, 28±3.9, 29±3.4, 30±2.8 & 34±3.9 mL at 0, 1500, 15000, 30000 & 50000 ppm, respectively) but only at 50000 ppm during wk 3 (36±5.3 *versus* 29±3.3 mL in the control; $p<0.01$). In females, mean water consumption was significantly higher ($p<0.05$ or 0.01) than the control at 30000 and 50000 ppm during wk 1 (27±3.8 & 31±6.1, respectively, *versus* 23±3.6 in the control) but only at 50000 ppm during wk 3 (32±6.0 *versus* 27±4.7 mL in the control; $p<0.05$).

TOXICOKINETICS

Toxicokinetic parameters for Advantame and Advantame-acid are summarised in Table 3.11, but given the analytical uncertainty, detailed comment on these values is not warranted.

In vitro data showed complete conversion of Advantame to Advantame-acid in SIF within 5 min (Section 3.2.2); based on the average metabolite ratio (i.e. AUC_{24} for Advantame ÷ AUC_{24} for Advantame-acid) calculated by FSANZ, it is estimated that a minimum of 90% of the administered dose of Advantame was converted to Advantame-acid prior to absorption. Given the likely overestimation of Advantame due to methodological limitations (by up to 5%), the actual level of GIT conversion was probably higher (i.e. >95%).

TABLE 3.11: MEAN C_{MAX} AND AUC_{24} VALUES FOR ADVANTAME AND ADVANTAME-ACID.

Dietary Level (ppm)	C_{max} (ng/mL)				AUC_{24} (ng.h/mL)			
	wk 1		wk 4		wk 1		wk 4	
	Males	Females	Males	Females	Males	Females	Males	Females
Advantame								
1500	54.1 (1)	201 (1)	612 (1)	413 (1)	777 (1)	1689 (1)	3509 (1)	3438 (1)
15000	127 (2.3)	158 (0.8)	220 (0.4)	499 (1.2)	1383 (1.8)	2695 (1.6)	2634 (0.8)	4799 (1.4)
30000	377 (7.0)	630 (3.1)	377 (0.6)	1043 (2.5)	3183 (4.1)	4412 (2.6)	2641 (0.8)	7245 (2.1)
50000	204 (3.8)	440 (2.2)	297 (0.5)	806 (2.0)	2541 (3.3)	4289 (2.5)	3814 (1.1)	8312 (2.4)
Advantame-acid								
1500	228 (1)	100 (1)	411 (1)	510 (1)	2786 (1)	1963 (1)	3855 (1)	4165 (1)
15000	1632 (7.2)	1485 (14.9)	1009 (2.5)	1435 (2.8)	25590 (9.2)	22587 (11.5)	17992 (4.7)	21454 (5.2)
30000	1695 (7.4)	2212 (22.1)	2197 (5.3)	3455 (6.8)	27708 (9.9)	32287 (16.4)	29065 (7.5)	38528 (9.2)
50000	2632 (11.5)	1348 (13.5)	2608 (6.3)	2763 (5.4)	34316 (12.3)	22863 (11.6)	32823 (8.5)	30412 (7.3)
Metabolite ratio[1]								
1500	-	-	-	-	3.6	1.2	1.1	1.2
15000	-	-	-	-	18.5	8.4	6.8	4.5
30000	-	-	-	-	8.7	7.3	11	5.3
50000	-	-	-	-	13.5	5.3	8.6	3.7

Results expressed as the mean, with the C_{max} or AUC_{24} ratios contained in parentheses; 1 = AUC_{24} for Advantame-acid ÷ AUC_{24} for Advantame

OPHTHALMOSCOPY

There were no treatment-related ophthalmoscopic abnormalities.

HAEMATOLOGY

In males, mean Hct and Hb were significantly lower ($p<0.01$ or 0.05) than the control across all treated groups (Hct: 0.478, 0.463, 0.456, 0.456 and 0.464 L/L[9] at 0, 1500, 15000, 30000 and 50000 ppm, respectively; Hb: 16.2, 15.6, 15.4, 15.4 and 15.6 g/dL[10] at 0, 1500, 15000, 30000 and 50000 ppm, respectively. In males, mean MCH was significantly lower than the control at 50000 ppm (19.2 *versus* 19.8 pg, respectively[11]). Mean monocytes counts and PT were also significantly lower than the control at 50000 ppm (0.17 *versus* 0.24 $x10^{-9}$/L[12] and 14.1 *versus* 15.0 sec[13], respectively). In females, mean WBC and lymphocytes were significantly lower ($p<0.01$ or 0.05) than the control across all treated groups (WBC: 6.73, 5.46, 5.18, 5.47 and 5.13 $x10^{-9}$/L at 0, 1500, 15000, 30000 and 50000 ppm, respectively; lymphocytes: 5.75, 4.62, 4.29, 4.58 and 4.16 $x10^{-9}$/L[14] at 0, 1500, 15000, 30000 and 50000 ppm, respectively).

There are several aspects to the above findings that argue against any treatment-related effect. Firstly, for those endpoints that were significantly different to the control across all or

[9] Normal range in 8-16 week old male Han Wistar rats = 0.396-0.525 L/L (CRL 2008)
[10] Normal range in 8-16 week old male Han Wistar rats = 13.7-17.6 g/dL (CRL 2008)
[11] Normal range in 8-16 week old male Han Wistar rats = 17.1-20.4 pg (CRL 2008)
[12] Normal range in 8-16 week old male Han Wistar rats = 0.03-0.18 x 10^{-9}/L (CRL 2008)
[13] Normal range in 8-16 week old male Han Wistar rats = 11.5-16.1 sec (CRL 2008)
[14] Normal range in 8-16 week old female Han Wistar rats = 0.82-5.66 10^{-9}/L (CRL 2008)

most doses (e.g. Hct, Hb, WBC and lymphocytes), no dose-response relationship was evident. Secondly, there was no consistency in the findings between males and females; the same endpoints were not significantly different to the control in both sexes. Age and sex-matched historical control data indicated that mean values for all treated groups were within the normal range of biological variation for each respective parameter. Indeed for some endpoints, the mean control value was outside the normal range (e.g. monocytes in males and lymphocytes in females) and therefore the significant difference may be attributable to the abnormal control value. In relation to the significantly lower Hct and Hb in males, there was no corroborative decrease in RBC. Based on this weight-of-evidence, none of the above statistically significant haematology findings are considered treatment-related.

CLINICAL CHEMISTRY

In males, mean plasma urea concentrations were significantly higher ($p<0.05$) than the control at and above 15000 ppm (6.26, 7.29, 8.16, 7.77 and 7.34 mmol/L at 0, 1500, 15000, 30000 and 50000 ppm, respectively). These statistical differences are not considered treatment-related as there was no dose-response relationship, no corroborative evidence of kidney dysfunction and no differences in females. The mean values were within the normal range for age- and sex-matched Han Wistar rats (4.39-8.78 mmol/L) (CRL 2008).

URINALYSIS

In females, specific gravity was significantly higher ($p<0.05$) than the control at 30000 and 50000 ppm (1057 and 1045 g/L, respectively, *versus* 1039 g/L), coincident with a non-significant reduction in urine volume (1.5 and 1.7 mL, respectively, *versus* 2.6 mL in the control). In the absence of a dose-response relationship and corroborative evidence from other endpoints (e.g. decreased water consumption – group mean water consumption was actually higher than the control), these findings are not considered treatment-related.

PATHOLOGY

Mean absolute and relative thymus weights of high-dose males were significantly lower ($p<0.01$) than the control (Absolute: 0.39 ± 0.095 *versus* 0.527 ± 0.088 g; Relative: 0.1582 ± 0.0322 *versus* 0.1932 ± 0.0233 %) but in the absence of any histopathology of this organ, unusual haematology or a similar effect in females, this finding was not considered toxicologically significant.

Other than the presence of pale material in the lower GIT of all high-dose females, there were no treatment-related macroscopic abnormalities. There was a treatment-related increase in the incidence of corticomedullary mineralisation of the kidneys in females (Table 3.12), which was statistically significant ($p<0.05$) at the highest dose. There were no other histopathological abnormalities. In the absence of a similar finding in males, that in the majority of rats the mineralisation was graded as minimal, that there was no corroborative evidence of kidney dysfunction and that mineralisation was not observed in studies of longer duration, this finding is not considered treatment-related.

TABLE 3.12: INCIDENCE OF CORTICOMEDULLARY MINERALISATION IN FEMALES

Parameter	Dietary level of Advantame (ppm)				
	0	1500	15000	30000	50000
N	10	10	10	10	10
Minimal	1	3	2	5	6
Slight	1	0	2	2	2
Total	2	3	4	7	8*

*p<0.05

Conclusion

The NOAEL following 4 weeks of dietary exposure to Advantame was 50000 ppm, the highest dietary concentration tested (equivalent to 6126 mg/kg bw/day in males and 6490 mg/kg bw/day in females), based on the absence of any toxicologically-significant effect at this dietary concentration.

3.2.4.2 Dogs

Barker MH et al (2002) ANS9801. Toxicity study by dietary administration to beagle dogs for 4 weeks. Report No. AJO 156/012585. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. **GLP**: UK, EC, OECD, US FDA & Japan. **QA statement**: Yes. **Guidelines**: Japan, US FDA & EC

*****Baldrey SF, Phelan MR, McBurney A & Holding J (2001) Pharmacokinetic report. Pharmacokinetics of ANS 9801 and ANS 9801-acid following a maximum tolerated dosage study by dietary administration to beagle dogs. Report No. AJO 156/012585.

Experimental

Advantame (99.2% purity; Batch No. 000530; sourced from the Sponsor) was admixed in the diet and fed to groups of 4 beagle dogs/sex at dietary concentrations of 0, 5000, 15000 or 50000 ppm for 4 weeks. These concentrations were chosen based on a maximum tolerated dose study (Report No. AJO 145/010022) where 50000 ppm was well tolerated for 2 weeks. Dogs were sourced from Harlan UK Ltd Hillcrest (England) and at the commencement of dosing were 23-27 weeks old and weighed 6.6-8.8 kg (females) or 5.9-9.1 kg (males). Dogs were offered 400 g of test or control diet each day, with water available *ad libitum*.

Observations for clinical signs were made at least twice daily, with more detailed observations made immediately before offering the diet, 0.5-2 h after offering the diet and at the end of the day. A physical examination was performed weekly. Bodyweight was recorded weekly. Food consumption was recorded daily. Ophthalmoscopy was performed prior to the commencement of dosing and during wk 4. ECG was performed prior to the commencement of dosing and during wk 2 and 4. Blood and urine samples were collected prior to the commencement of dosing and during wk 4 for the analysis of standard haematology, clinical chemistry and urinalysis parameters, (Appendix 1) with the exclusion of CPK and inclusion of phospholipids. Blood samples were also collected on d 1 and 27 at 0, 0.5, 1, 3, 6, 12 and 24 h after offering the diet for toxicokinetic analysis; concentrations of Advantame and Advantame-acid were analysed by LC-MS/MS. At the end of the treatment period, dogs were killed and necropsied. Standard organs and tissues were weighed (in addition to the lungs and salivary glands) and histopathologically examined (Appendix 2).

Results

MORTALITIES & CLINICAL SIGNS

There were no deaths. Pale faeces were noted in all dogs at 50000 ppm starting from wk 1, which the authors suggested was due to the presence of the test material rather than a toxicological effect. As Advantame and Advantame-acid are white in colour, this suggestion seems reasonable, particularly as there were no other findings (such as any abnormalities of the liver/gall bladder) that would provide an alternate explanation for the pale faeces.

BODYWEIGHT & FOOD CONSUMPTION

There was no treatment-related effect on mean absolute bodyweight. In both sexes, mean

bodyweight gain over the 4-week treatment period was lower than the control at 50000 ppm, with the difference in females statistically significant ($p<0.5$) (1.2/1.2, 1.3/0.9, 0.8/1.1 and 0.7/0.7 kg in males/females at 0, 5000, 15000 and 50000 ppm, respectively). As there was no treatment-related effect on food consumption, the lower bodyweight gain, particularly at the highest dose, is considered to reflect the relatively high concentration of a non-caloric substance in the diet rather than to a direct toxicological effect. This conclusion is supported by the reduction in food conversion efficiency (g bw gain/g food consumed)[15], which mainly occurred at the highest dose (0.43, 0.46, 0.29 and 0.26 in males and 0.43, 0.32, 0.39 and 0.25 in females at 0, 5000, 15000 and 50000 ppm, respectively).

Mean achieved doses of Advantame over four weeks were 232/254, 737/743 and 2385/2488 mg/kg bw/day in males/females at 5000, 15000 and 50000 ppm, respectively.

TOXICOKINETICS

Mean C_{max} and AUC_{24} values for Advantame and Advantame-acid are summarised in Table 3.13, with both detectable in all blood samples collected on d 1 and 27. Based on analytical uncertainty, these values are viewed as estimates only but qualitatively suggest dose-dependent, non-linear kinetics. There was no consistent difference between males and females. Systemic exposure was generally higher at d 27 than 1. Exposure to Advantame-acid was higher than Advantame. Based on the average metabolite ratio, it is estimated that a minimum of 97% of the administered dose of Advantame was converted to Advantame-acid prior to absorption. Given the likely overestimation of Advantame due to analytical uncertainty, the actual level of GIT conversion is probably closer to 100%.

TABLE 3.13: C_{MAX} AND AUC_{24} VALUES FOR ADVANTAME AND ADVANTAME-ACID.

Parameter	5000 ppm		15000 ppm		50000 ppm	
	Males	***Females***	***Males***	***Females***	***Males***	***Females***
Advantame						
C_{max} (ng/mL)						
d 1	777 (1)	332 (1)	591 (0.8)	608 (1.8)	1246 (1.6)	2958 (8.9)
d 27	612 (1)	1867 (1)	3149 (5.1)	3913 (2.1)	2071 (3.4)	4542 (2.4)
AUC_{24} (ng.h/mL)						
d 1	15841 (1)	4662 (1)	11457 (0.7)	10711 (2.3)	18521 (1.2)	54008 (11.6)
d 27	11281 (1)	34926 (1)	56388 (5.0)	78658 (2.3)	37308 (3.3)	74019 (2.1)
Advantame-acid						
C_{max} (ng/mL)						
d 1	13509 (1)	18184 (1)	22562 (1.7)	24431 (1.3)	61023 (4.5)	74264 (4.1)
d 27	35928 (1)	57661 (1)	109643 (3.1)	57985 (1.0)	71015 (2.0)	59778 (1.0)
AUC_{24} (ng.h/mL)						
d 1	215013 (1)	312955 (1)	399652 (1.9)	396956 (1.3)	900146 (4.2)	1310989 (4.2)
d 27	677423 (1)	877985 (1)	1845643 (2.7)	887608 (1.0)	1061670 (1.6)	994634 (1.1)
Metabolite ratio[1]						
d 1	13.0	73.6	34.9	37.7	47.2	26.5
d 27	58.9	25.5	33.0	11.1	27.7	12.9

Results are expressed as the mean, with the C_{max} and AUC_{24} ratios contained in parentheses; 1 = AUC_{24} for Advantame-acid ÷ AUC_{24} for Advantame.

OPHTHALMOSCOPY & ECG

Ophthalmoscopy and ECG were unremarkable.

[15] Calculated by FSANZ

HAEMATOLOGY

To aid in the comparison with findings from other studies, selected haematology parameters are summarised in Table 3.14. There was no treatment-related effect on any haematology parameter.

TABLE 3.14: SELECTED HAEMATOLOGY FINDINGS

Parameter	0 ppm		5000 ppm		15000 ppm		50000 ppm,	
	Male	Female	Male	Female	Male	Female	Male	Female
Hct (L/L)								
Pretreatment	0.374	0.394	0.360	0.400	0.395	0.402	0.372	0.393
wk 4	0.389	0.414	0.391	0.426	0.412	0.441	0.386	0.410
Hb (g/dL)								
Pretreatment	12.5	13.4	12.2	13.6	13.3	13.6	12.6	13.3
wk 4	12.6	13.4	12.7	13.8	13.4	14.2	12.7	13.3
RBC ($x10^{-12}$/L)								
Pretreatment	5.58	5.92	5.38	5.81	5.80	5.80	5.53	5.78
wk 4	5.87	6.20	5.87	6.19	6.05	6.40	5.86	6.17
Reticulocytes (%)								
Pretreatment	0.57	0.70	0.71	0.81	0.50	0.64	0.53	0.60
wk 4	0.65	0.67	0.67	1.09	0.67	0.79	0.56	0.61
WBC ($x10^{-9}$/L)								
Pretreatment	10.06	11.26	12.08*	10.61	11.39	12.93	9.56	12.74
wk 4	10.75	11.97	12.95	13.33	15.38	13.74	12.541	11.23
Lymphocytes ($x10^{-9}$/L)								
Pretreatment	3.70	4.12	4.37	4.18	4.60	4.42	3.43	4.56
wk 4	3.38	3.82	4.34	4.28	4.80*	4.61	4.14*	4.10

Results are expressed as the mean; *p<0.05

CLINICAL CHEMISTRY

Selected clinical chemistry findings in males are summarised in Table 3.15. Mean plasma urea concentrations during wk 4 were elevated in treated groups relative to the control, reaching statistical significance at 15000 and 50000 ppm ($p<0.05$). These findings are unlikely to be treatment-related for the following reasons: (1) there was no dose-response relationship; (2) pre-treatment urea concentrations were already higher than the control; and (3) urea was increased across all groups (including the control) relative to pre-treatment values (both on a group and individual dog basis) by up to 1.5, 1.5, 1.6 and 1.4-fold at 0, 5000, 15000 and 5000 ppm, respectively.

Mean glucose concentrations at wk 4 were significantly lower ($p<0.05$) than the control across all treated male groups but in the absence of a dose-response relationship and that there was little difference between each groups' mean pre-treatment glucose concentration and the wk 4 concentration, this finding is not considered treatment-related. Significantly elevated ($p<0.01$ or 0.05) plasma sodium and chloride concentrations in 50000 ppm females during wk 4 were not considered treatment-related as pre-treatment values for both parameters were already significantly higher than the control.

TABLE 3.15: SELECTED CLINICAL CHEMISTRY FINDINGS

Parameter	0 ppm		5000 ppm		15000 ppm		50000 ppm,	
	Male	Female	Male	Female	Male	Female	Male	Female
Urea (mmol/L)								
Pretreatment	3.08	3.45	3.30	3.62	3.58	4.18	3.86	3.47
wk 4	3.74	4.86	4.62	4.76	5.11*	4.58	4.97*	4.48
Glucose (mmol/L)								
Pretreatment	6.02	5.81	5.75	5.97	6.09	5.98	6.18	5.88
wk 4	6.23	5.74	5.78*	6.16	5.87*	5.86	5.91*	5.96

Results expressed as the mean; *p<0.05

URINALYSIS

There was no treatment-related effect on any urinalysis parameter.

ORGAN WEIGHTS

Selected organ weight findings are summarised in Table 3.16. Marginally higher mean absolute brain weight was noted across all treated groups, reaching statistical significance in 50000 ppm males. Given the small magnitude of the difference between treated and control brain weights (i.e. <15%), the absence of a dose-response relationship or any detectable brain pathology, and as the statistical difference is likely attributable to a single male with a high brain weight value (90.5 g), this result is not considered treatment-related.

Mean absolute thymus weights were lower than the control across all treated groups, with the differences statistically significant in females (p<0.01 or 0.05). Relative thymus weights were also lower than the control. While the magnitude of the decrease was moderate, the absence of a clear dose-response relationship or corroborative evidence from other endpoints (e.g. decreased size; abnormal architecture or histopathology; haematology) indicates that the decreased thymus weights were not treatment-related.

TABLE 3.16: SELECTED ORGAN WEIGHT FINDINGS

Organ	0 ppm		5000 ppm		15000 ppm		50000 ppm,	
	Male	Female	Male	Female	Male	Female	Male	Female
Brain (g) (absolute)	74.0	69.4	79.2 **(+7%)**	76.7 **(+11%)**	79.4 **(+7%)**	71.4 **(+3%)**	83.3* **(+13%)**	76.3 **(+10%)**
Thymus (g) (absolute)	17.44	20.74	17.39 **(-2%)**	16.81* **(-19%)**	10.82 **(-38%)**	16.00* **(-23%)**	12.37 **(-29%)**	15.28** **(-24%)**
Thymus (%) (relative)[1]	0.20	0.23	0.19 **(-5%)**	0.20 **(-13%)**	0.13 **(-35%)**	0.18 **(-22%)**	0.14 **(-30%)**	0.18 **(-22%)**

Results expressed as the mean, with the % increase (+) or decrease (-) relative to the control contained in parentheses; *p<0.05 ; 1 = mean relative organ weights were calculated by FSANZ as these were not contained in the study report; no statistical analysis was performed on the relative organ weight data

MACRO AND HISTOPATHOLOGY

There were no treatment-related macroscopic or histopathological abnormalities.

Conclusions

The NOAEL in dogs following 4-weeks of dietary exposure to Advantame was 50000 ppm (equal to 2385 mg kg bw/day in males and 2488 mg/kg bw/day in females), the highest dietary concentration tested.

3.2.5 Subchronic Toxicity Studies

3.2.5.1 Mice

Chase K (2002b) ANS9801. Toxicity study by dietary administration to CD-1 mice for 13 weeks. Report No. AJO 174/014348. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. **GLP**: UK, EC, OECD, US FDA & Japan. **QA statement**: Yes.

***** Baldrey SF, Flack I & Carr M (2002f) Toxicokinetic report. ANS9801. Toxicity study by dietary administration to CD-1 mice for 13 weeks. Report No. AJO 174/014348. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. **GLP**: UK, EC, OECD, US FDA & Japan. **QA statement**: Yes.

Experimental

This study was specifically designed as a range-finding study for a 2-year carcinogenicity study (Horne 2006) and therefore did not analyse haematology, clinical chemistry or urinalysis parameters. In addition, no histopathology or ophthalmoscopy were performed.

Advantame (99.5% purity; Batch No. 001227; sourced from the Sponsor) was admixed in the diet and fed *ad libitum* to groups of twenty CD-1 mice/sex at concentrations of 0, 5000, 15000 or 50000 ppm for 13 weeks. Mice were sourced from Charles River (UK), were approximately 40-44 days old and weighed 32 (males) or 35 g (females) at the commencement of dosing. Satellite groups of 40 mice/sex were dosed similarly for toxicokinetic analysis. Observations for mortalities and clinical signs were made daily, with a more detailed examination performed weekly. Body weight and food consumption were recorded at weekly intervals. Blood samples were collected during wk 1, 6 and 12 from the satellite groups for the measurement of Advantame and Advantame-acid by LC-MS/MS; standard toxicokinetic parameters were recorded or calculated. Mice were killed at the end of the treatment period and necropsied. Standard organs were weighed (except for the thyroid) (Appendix I).

Results

There were no treatment-related mortalities or clinical signs.

Mean bodyweight gain over the 13 weeks of treatment was ~10% lower than the control at 50000 ppm (95/90, 99/105 and 89/90% of the control in males/females at 5000, 15000 and 50000 ppm, respectively) but not significantly. There was no difference in food consumption between treated and control groups. Food conversion efficiency varied over time and between doses, and overall was lower at 50000 ppm compared to the control (93/94, 97/111 and 83/89% of the control at 5000, 15000 and 50000 ppm, respectively). The apparent effect on bodyweight gain and food conversion efficiency at the highest dose is probably due to the relatively high concentration of a non-caloric substance in the diet rather than to a direct toxicological effect; this is consistent with observations from other laboratory animal studies.

Mean achieved doses of Advantame over thirteen weeks were 734/892, 2129/2593 and 7444/9317 mg/kg bw/day in males/females at 5000, 15000 and 50000 ppm, respectively. Toxicokinetic parameters for Advantame and Advantame-acid are summarised in Table 3.17. The kinetics appeared to be linear over the dose range as shown by the proportional increase in C_{max} and AUC_{24} values. Systemic exposure to Advantame-acid was higher than to the parent compound. Exposure was generally higher in females than males. Based on the average metabolite ratio (calculated by FSANZ), it is estimated that a minimum of 90% of the administered dose of Advantame was converted to Advantame-acid prior to absorption.

Given the likely overestimation of Advantame due to analytical uncertainty, the actual level of GIT conversion was probably higher (i.e. >95%).

TABLE 3.17: C_{MAX} AND AUC_{24} VALUES FOR ADVANTAME AND ADVANTAME-ACID

Parameter	5000 ppm		15000 ppm		50000 ppm	
	Males	***Females***	***Males***	***Females***	***Males***	***Females***
Advantame						
C_{max} (ng/mL)						
wk 1	106 (1)	135 (1)	267 (2.5)	342 (2.5)	615 (5.8)	1253 (9.3)
wk 6	75.9 (1)	65.1 (1)	167 (2.2)	319 (4.9)	217 (2.9)	342 (5.3)
wk 12	176 (1)	167 (1)	169 (1.0)	1514 (9.1)	828 (3.6)	635 (3.8)
AUC_{24} (ng.h/mL)						
wk 1	1258 (1)	1824 (1)	4149 (3.3)	4844 (2.7)	7812 (6.2)	12431 (6.8)
wk 6	751 (1)	849 (1)	2340 (3.1)	3351 (3.9)	3825 (5.1)	6196 (7.3)
wk 12	1807 (1)	2294 (1)	2517 (1.4)	9065 (4.0)	6268 (3.5)	9125 (4.0)
Advantame-acid						
C_{max} (ng/mL)						
wk 1	670 (1)	711 (1)	1172 (1.7)	2327 (3.3)	4907 (7.3)	7659 (10.8)
wk 6	442 (1)	783 (1)	1292 (2.9)	2358 (3.0)	5617 (12.7)	8416 (10.7)
wk 12	338 (1)	697 (1)	1159 (3.4)	1946 (2.8)	5818 (17.2)	6655 (9.5)
AUC_{24} (ng.h/mL)						
wk 1	12207 (1)	13811 (1)	25591 (2.1)	42433 (3.1)	90548 (7.4)	136701 (9.9)
wk 6	8602 (1)	12704 (1)	26624 (3.1)	46231 (3.6)	95680 (11.1)	141734 (11.2)
wk 12	6518 (1)	10953 (1)	23133 (3.5)	36826 (3.4)	97421 (14.9)	138790 (12.7)
Metabolite ratio[1]						
wk 1	9.7	7.6	6.2	8.8	11.6	11
wk 6	11.5	15	11.4	13.8	17.2	22.9
wk 12	3.6	4.8	9.2	4.1	15.5	15.2

Results are expressed as means, with the C_{max} or AUC_{24} ratio contained in parentheses; 1 = AUC_{24} for Advantame-acid ÷ AUC_{24} for Advantame.

OTHER FINDINGS

There was no treatment-related effect on absolute or relative organ weights. In males, there was an increase in the incidence of congestion of the lungs across all treated groups relative to the control (5, 15, 32 and 30% at 0, 5000, 150000 and 50000 ppm, respectively) but in the absence of a similar trend in females (40, 15, 15 and 40%, respectively), a dose-response relationship or statistical significance, this finding was not considered treatment-related.

Conclusion

There were no adverse effects observed at 50000 ppm (equivalent to 7444 mg/kg bw/day in males and 9317 mg/kg bw/day in females), the highest dietary concentration tested.

3.2.5.2 Rats

Chase K et al (2004) ANS9801. Toxicity study by dietary administration to Han Wistar rats for 13 weeks followed by a 4 week recovery period. Report No. AJO 176/014075. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. **GLP**: UK, EC, OECD, US FDA & Japan. **QA statement**: Yes.

***** Baldrey SF, Phelan MR, Holding J, Carr MJ & Cook SC (2004) Toxicokinetic report. ANS9801. Toxicity study by dietary administration to Han Wistar rats for 13 weeks followed by a 4 week recovery period. Report No. AJO 176/014075. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. **GLP**: UK, EC, OECD, US FDA & Japan. **QA statement**: Yes.

Wing MG (2004) ANS9801. Immunology Report. Toxicity study by dietary administration to Han Wistar rats for 13 weeks followed by a 4 week recovery period. Report No. AJO 176/014075. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. **GLP**: UK, EC, OECD, US FDA & Japan. **QA statement**: Yes.

Experimental

Advantame (99.5% purity; Batch No. 001227; sourced from the Sponsor) was admixed in the diet and fed to groups of 20 rats/sex (HsdBrl Han: Wist strain) at dietary concentrations of 0, 1500, 5000, 15000 or 50000 ppm for 13 weeks. An additional 5 rats/sex were included in the control, 15000 and 50000 ppm groups and allowed a 4-week recovery period following the 13 weeks of treatment. Satellite groups of 9 rats/sex were used for toxicokinetic analysis. Rats were sourced from Charles River UK Ltd (Kent, England). At the commencement of dosing, males were ~38-42 days old and weighed 123-145 g, while females were ~42-44 days old and weighed 112-129 g. Rats were housed individually under standard conditions, with food and water available *ad libitum*.

Rats were observed at least twice daily for clinical signs, with a more detailed examination performed weekly. Bodyweight and food consumption were recorded weekly. Water consumption over 3 days was recorded during wk 1, 5 and 11 of treatment, and wk 3 of the recovery period. Ophthalmoscopy was performed on all rats prior to treatment and in the control and 50000 ppm groups during wk 12. A functional observational battery (FOB) was performed on 10 rats/sex from the control, 15000 and 50000 ppm groups during wk 13 of treatment and wk 3 of the recovery period.

Blood samples were obtained at various times during the treatment and recovery periods for the analysis of the standard haematology and clinical chemistry parameters (Appendix 1) (wk 13 of treatment and wk 4 of recovery), immunotoxicology (10 rats/group at wk 4 and 13 of treatment and wk 4 of recovery) and toxicokinetics (satellite groups at wk 1 and 13). The immunotoxicity of Advantame was assessed because reduced thymus weights were observed in a 4-week dietary study (see Section 3.2.4). The proportions of different cell populations were analysed by flow cytometry, while lymphocyte proliferation was assessed via a mitogen assay.

Overnight urine was collected during wk 12 of treatment and wk 3 of the recovery period, and analysed for the standard urinalysis parameters (Appendix 1). Following sacrifice, rats were necropsied and their organs weighed (Appendix 2, in addition to the lungs, pituitary and salivary gland). Histopathology was performed on the standard range of tissues/organs (Appendix 2) with the addition of the tongue and exception of the Zymbal's gland and any gross lesions.

Results

MORTALITIES & CLINICAL SIGNS

There were no treatment-related mortalities or clinical signs, noting that the faeces of rats in the 15000 and 50000 ppm groups appeared pale due likely to the high concentration of test material. Light or dark green, or light or dark pink staining of the cage lining was noted for the occasional treated rat, which the authors indicated was associated with the contact of faeces with the lining. During wk 2 of the recovery period, overactivity was observed in females in the 15000 and 50000 ppm groups (2 and 4/5 rats, respectively). In the absence of similar observations at other times this observation is not considered treatment-related.

BODYWEIGHT & FOOD CONSUMPTION

Bodyweight, bodyweight gain, food consumption and food conversion efficiency were unaffected by treatment. There was a dose-related increase in water consumption, which was statistically significant ($p<0.05$ or 0.01) in males at 50000 ppm during wk 5 (113% of the control) and at and above 5000 ppm during wk 11 (110, 115 and 128% of the control, respectively). In females, water consumption was significantly higher ($p<0.05$ or 0.01) than the control at and above 15000 ppm during wk 5 and 11 (~130% of the control). Elevated water consumption was not observed in any other study.

Mean achieved doses of Advantame over thirteen weeks were 118/146, 415/481, 1271/1487 and 4227/5109 mg/kg bw/day in males/females at 1500, 5000, 15000 and 50000, respectively.

TOXICOKINETICS

C_{max} and AUC_{24} values for Advantame and Advantame-acid are summarised in Table 3.18. Based on analytical uncertainty, detailed comment on these data is not warranted. There was a dose-related increase in systemic exposure to Advantame and Advantame-acid, which was generally not proportional to the dose increment. Exposure to Advantame-acid was higher than to Advantame. Based on the average metabolite ratio (calculated by FSANZ), it is estimated that a minimum of 81% of the administered dose of Advantame was converted to Advantame-acid prior to absorption. Given the likely overestimation of Advantame due to methodological limitations, the actual level of GIT conversion was probably higher (i.e. >86%).

TABLE 3.18: C_{MAX} AND AUC_{24} VALUES FOR ADVANTAME & ADVANTAME-ACID

Dietary level (ppm)	wk 1		wk 13	
	Males	Females	Males	Females
C_{max} (ng/mL) Advantame				
1500	80 (1)	155 (1)	82 (1)	139 (1)
5000	85 (1.1)	133 (0.86)	69 (0.84)	153 (1.1)
15000	350 (4.4)	251 (1.6)	471 (5.7)	896 (6.4)
50000	344 (4.3)	978 (6.3)	1132 (13.8)	358 (2.6)
AUC_{24} (ng.h/mL) Advantame				
1500	644 (1)	1117 (1)	720 (1)	1051 (1)
5000	669 (1)	1003 (0.9)	620 (0.86)	1163 (1.1)
15000	2440 (3.8)	2653 (2.4)	5675 (7.9)	8680 (8.3)
50000	4222 (6.6)	6754 (6.0)	8312 (11.5)	4091 (3.9)
C_{max} (ng/mL) Advantame-acid				
1500	218 (1)	213 (1)	214 (1)	208 (1)
5000	413 (1.9)	413 (1.9)	260 (0.8)	282 (1.4)
15000	1664 (7.6)	3921 (18.4)	732 (3.4)	697 (3.4)

Dietary level (ppm)	wk 1		wk 13	
	Males	Females	Males	Females
50000	3612 (16.6)	3145 (14.8)	1646 (7.7)	1759 (8.5)
AUC_{24} (ng.h/mL) Advantame-acid				
1500	2654 (1)	2183 (1)	2510 (1)	2015 (1)
5000	5548 (2.1)	4820 (2.2)	3095 (1.2)	4483 (2.2)
15000	20271 (7.6)	25994 (11.9)	10427 (4.2)	10325 (5.1)
50000	52603 (19.8)	46427 (21.3)	26027 (10.4)	25687 (12.7)
Metabolite ratio[1]				
1500	4.1	2.0	3.5	1.9
5000	8.3	4.8	5	3.9
15000	8.3	9.8	1.8	1.2
50000	12.4	6.9	3.1	6.3

Results expressed as the mean, with the achieved dose, C_{max} and AUC_{24} ratios contained in parentheses; 1 = AUC_{24} for Advantame-acid ÷ AUC_{24} for Advantame

IMMUNOTOXICITY

In females during wk 4, total B cells were significantly lower ($p<0.05$) than the control at and above 5000 ppm (33±7.2, 29.4±5.1, 26.7±6.2, 26.3±4.7, 28.4±4.9% at 0, 1500, 5000, 15000 and 50000 ppm, respectively). In males during wk 4, total T cells were significantly lower ($p<0.05$ or 0.01) than the control at every dietary concentration (46.7±4.4, 38.4±10.7, 34.4±6.6, 41.2±6.2 and 35.1±5.3% at 0, 1500, 5000, 15000 and 50000 ppm, respectively); only the 50000 ppm group had significantly lower total T cells during wk 13. This finding is consistent with the reduction in lymphocytes observed during haematology in the main study. In contrast, females had significantly higher ($p<0.05$) total T cells during wk 13 at 150000 and 50000 ppm (64.4±9.1 and 61.0±8.2%, respectively, *versus* 55.0±9.5% in the control). During wk 13, CD4+ and CD8+ T cells were significantly higher ($p<0.05$ or 0.01) than the control at and above 5000 ppm in females, but significantly lower ($p<0.01$) than the control in males at 50000 ppm. Based on the absence of a dose-response relationship for any of these findings, the lack of consistency between sexes (in terms of the cell populations potentially affected and the direction of the change) and over time, and the absence of any pathology of the thymus or spleen, none are considered treatment-related.

In males during wk 4, stimulation of peripheral T lymphocytes at two different concentrations of concanavalin A (conA) was significantly lower ($p<0.05$ or 0.01) than the control in all treated groups (Table 3.19); this reduction in T cell responsiveness was consistent with the reduction in total T cells described above. No such affect was observed during wk 13 or in females at any time. In the absence of a dose-response relationship and given the large variation of the data, none of these findings are considered to suggest that Advantame is immunotoxic.

TABLE 3.19: RESULTS OF MITOGEN ASSAY (WEEK 4)

Dietary level (ppm)	ConA (1.25 µg/mL)		ConA (5 µg/mL)	
	Male	Female	Male	Female
0	28.7±24.6	17.3±14.2	361.3±157.7	201.9±110.9
1500	8.9±6.0**	18.2±15.9	160.0±109.0*	313.8±243.9
5000	11.1±6.0**	14.1±20.7	205.3±81.5*	155.0±143.1
15000	9.3±3.7**	14.0±14.0	293.3±105.6*	138.0±112.0
50000	13.7±14.5**	32.6±29.8	203.9±120.4**	236.5±166.1

Results expressed as the mean stimulation index (SI) = mean mitogen counts per min (cpm) ÷ medium control

OPHTHALMOSCOPY

There were no treatment-related ophthalmoscopic abnormalities.

HAEMATOLOGY

Selected haematology parameters at wk 13 of treatment and wk 4 of the recovery phase are summarised in Table 3.20. In both sexes during wk 13, mean Hct and Hb were significantly lower ($p<0.05$ or 0.01) than the control at 50000 ppm, and in females at 15000 ppm ($p<0.01$). Co-incidentally, mean RBC was significantly lower ($p<0.05$) than the control in high-dose females. Analysis of reticulocytes was conducted by light microscopy, which could only quantify counts at and above 2%. As the majority of rats across all groups (including the control) had reticulocytes counts recorded as <2%, it is not possible to determine the effect of treatment on this parameter and whether there was a compensatory increase in reticulocytes due to the lower RBC in high-dose females. While the magnitude of these significant differences in Hb, Hct and RBC is quite small (2-4% lower than the concurrent control), the absence of significant differences at the end of the recovery phase is suggestive of a treatment-related effect. However, as all values were within the reference range for age- and sex matched rats of the same strain, these treatment-related differences are considered to be in the normal range of biological variation and are therefore not interpreted as adverse.

During wk 13, there was a dose-related reduction in WBC in males, which was statistically significant at 15000 and 50000 ($p<0.01$), and likely attributable to the significant ($p<0.01$) reduction in lymphocytes. In 50000 ppm females, mean lymphocyte counts were also significantly lower ($p<0.01$) than the control. The absence of significant differences between these groups and the control during the recovery phase suggests that these reductions are treatment-related. However, it is important to note that these endpoints are normally highly variable and given that all values fell within the respective reference ranges, they are not considered toxicologically significant.

In 50000 ppm females, monocyte counts were significantly lower ($p<0.05$) than the control during wk 13 but not during the recovery phase. This finding is not considered treatment-related as the mean (0.09 $x10^9$/L) was identical that of the 15000 ppm group during the recovery phase, which was not significantly different to the control group.

At 50000 ppm, mean large unstained cells (LUC) were significantly lower ($p<0.01$) than the control in both sexes, in addition to 15000 ppm males. As reductions in LUC are not clinically relevant, these significant differences are considered incidental findings.

In females during wk 13, mean prothrombin time (PT) was significantly lower ($p<0.01$) than the control at 15000 and 50000 ppm. In 50000 ppm males, mean activated partial thromboplastin time (APTT) was significantly lower than the control. There was no significant difference in these parameters following 4-weeks of recovery. As the magnitude of these differences is less than variability in the control group between wk 13 *versus* recovery wk 4, and as these values fall within the normal reference range, these statistically significant differences are not considered treatment-related.

TABLE 3.20: SELECTED HAEMATOLOGY FINDINGS

Parameter	Dietary concentration (ppm)									
	0		1500		5000		15000		50000	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Hb (g/dL)[1]										
Wk 13	16.8	15.9	16.8	15.8	16.6	15.7	16.7	15.4**	16.5*	15.2**
Recovery	16.4	16.0	-	-	-	-	16.1	16.1	16.6	15.9
Hct (L/L)[2]										
Wk 13	0.479	0.448	0.481	0.447	0.474	0.443	0.477	0.435**	0.468**	0.432**
Recovery	0.473	0.464	-	-	-	-	0.467	0.464	0.481	0.464

Parameter	Dietary concentration (ppm)									
	0		1500		5000		15000		50000	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
RBC[3] ($x10^{12}$/L)										
Wk 13	9.02	8.24	9.08	8.16	9.09	8.16	9.06	8.01	8.93	8.03*
Recovery	9.01	8.04	-	-	-	-	9.02	8.14	9.03	8.27
WBC[4] ($x10^9$/L)										
Wk 13	7.55	4.10	7.35	4.27	6.73	3.92	6.18**	3.61	6.07**	3.43
Recovery	5.70	2.96	-	-	-	-	5.65	3.52	6.30	3.63
Lymphocytes ($x10^9$/L)[5]										
Wk 13	6.08	3.42	5.89	3.48	5.32	3.20	4.74**	3.00	4.68**	2.74**
Recovery	4.46	2.16	-	-	-	-	4.63	2.88	5.1	2.79
Monocytes ($x10^9$/L)[6]										
Wk 13	0.22	0.11	0.19	0.11	0.22	0.10	0.21	0.10	0.20	0.09*
Recovery	0.21	0.10	-	-	-	-	0.16	0.09	0.18	0.13
LUC ($x10^9$/L)[7]										
Wk 13	0.07	0.03	0.06	0.03	0.05	0.02	0.05*	0.02	0.04**	0.02**
Recovery	0.06	0.03	-	-	-	-	0.07	0.02	0.06	0.02
PT (sec)[8]										
Wk 13	13.7	14.6	13.6	14.1	13.9	14.7	13.9	13.8**	13.6	13.7**
Recovery	15.5	14.2	-	-	-	-	13.8**	13.4	13.9**	14.2
APTT (sec)[9]										
Wk 13	20.8	18.3	19.7	16.8	20.2	18.0	19.3	16.8	18.5**	17.5
Recovery	26.3	22.2	-	-	-	-	22.6	17.8	21.9	19.1

Results expressed as the mean; *p<0.05; **p<0.01
1 = Reference range: 13.6-17.4 g/dL in males & 13.7-17.2 g/dL in females (CRL 2008)
2 = Reference range: 0.385-0.52 L/L in males & 0.385-0.492 L/L in females (CRL 2008)
3 = Reference range: 7.62-9.99 $x10^{12}$/L in males & 7.16-9.24 $x10^{12}$/L (CRL 2008)
4 = Reference range: 1.98-11.06 $x10^9$/L in males & 0.96-7.88 $x10^9$/L (CRL 2008)
5 = Reference range: 1.19-9.45 $x10^9$/L in males & 0.68-6.8 $x10^9$/L (CRL 2008)
6 = Reference range: 0.03-0.27 $x10^9$/L in males & 0.01-0.13 $x10^9$/L (CRL 2008)
7 = Reference range: 0-0.07 $x10^9$/L in males & 0-0.05 $x10^9$/L (CRL 2008)
8 = Reference range: 11.55-16.1 sec in males & 11.3-15.2 sec (CRL 2008)
9 = Reference range: 17.4-34.4 sec in males & 16.3-36.2 sec (CRL 2008)

CLINICAL CHEMISTRY

In both sexes during wk 13, significantly lower bilirubin (p<0.01) was determined across most doses (with the exception of low-dose females) (males: 2 µmol/L in all treated groups *versus* 3 µmol/L in the control; females: 2 µmol/L at and above 5000 ppm *versus* 3 µmol/L in the control). In the absence of a dose-response relationship, that differences in males were still evident following 4-weeks of recovery and as decreased bilirubin is not normally clinically relevant, these significant differences are not considered attributable to treatment.

Other significant differences (p<0.01) in treated males at the majority of doses included lower mean K, Ca, P, total protein, albumin and A/G ratio. For Ca and P, differences remained following 4 weeks of recovery, which argues against a treatment-related effect. In females, Ca was also significantly lower (p<0.01) than the control at 15000 and 50000 ppm (2.75 and 2.74 mmol/L *versus* 2.82 mmol/L in the control). As none of the findings followed a dose-response relationship and that differences were of a small magnitude, none are considered treatment-related.

In females, mean urea was significantly lower (p<0.01 or 0.01) than the control at 15000 and 50000 ppm (7.29, 6.83, 7.02, 6.45 and 6.60 mmol/L at 0, 1500, 5000, 15000 and 50000

ppm, respectively) during wk 13 but not after 4 weeks of recovery (6.41, 6.57 and 6.95 mmol/L at 0, 15000 and 50000 ppm, respectively). The lower plasma urea concentrations may reflect the reduced intake of dietary protein because of the presence of Advantame in the diet and is consistent with results of the chronic rat study (Horne et al 2005) where reduced urea occurred in both sexes at 50000 ppm. This finding is considered to be a treatment-related adaptive response and hence not adverse, which is supported by the absence of corroborative evidence of liver dysfunction and as all values fell within the normal range for female Han Wistar rats aged 17-weeks or older [4.18-8.93 mmol/L (CRL 2008)].

URINALYSIS

Urinary parameters were unremarkable in males.

In females during wk 12, mean urine volume, Na, K and Cl were significantly lower ($p<0.01$ or 0.05) than the control at and above 5000 ppm. These parameters remained lower than the control at the highest dose during wk 3 of the recovery period, but not significantly so. Given the large normal variation inherent in these parameters, the lack of a dose-response relationship and absence of associated changes in serum Na, K or Cl, these findings are not considered treatment-related.

ORGAN WEIGHTS AND PATHOLOGY

Absolute and relative organ weights were unremarkable. The only treatment-related macroscopic abnormality was the occurrence of pale contents in the caecum and colon at 50000 ppm (14/20 and 18/20 in males/females, respectively, relative to 0/20 in the controls); this results was statistically significant ($p<0.001$) and considered to reflect the high concentration of test material in the diet.

Histopathology of rats sacrificed at wk 13 (n=19 or 20) revealed an increased incidence of degenerate fibres of the optic nerve at 50000 ppm in males (20% *versus* 10% in the control) and at and above 15000 ppm in females (15%, 20%, 25%, 37% and 55% at 0, 1500, 5000, 15000 and 50000 ppm, respectively), with the result at 50000 ppm statistically significantly ($p<0.01$) in females. In the groups sacrificed after 4 weeks of recovery (n=5), there was no difference in the incidence of degenerate fibres of the optic nerve between treated and control rats (40/40%, 0/0%, 0/0%, 0/80%, 20/40% in males/females at 0, 1500, 5000, 15000 and 50000 ppm, respectively). In the absence of any corroborative evidence, including abnormal ophthalmoscopy or neuropathology at other sites, this finding is most likely a consequence of blood sampling via the retro-orbital sinus rather than a treatment-related effect.

There was an increased incidence of mineralisation of the medulla of the kidneys in high-dose females (8/20 *versus* 13/20 in the control; graded as minimal), which was not statistically significant or evident following the 4-week recovery period. In the absence of suitable historical control data for this finding, its toxicological significance is unclear. The incidence of corticomedullary mineralisation of the kidneys (graded as minimal) was elevated in females at 15000 and 50000 ppm (4/5) relative to the control (1/5) at the end of the 4-week recovery period; in the absence of this finding during the treatment period (i.e. wk 13), it is not considered treatment-related. Further, the authors stated that corticomedullary mineralisation of the kidneys is a common age-related finding in female rats of this strain.

There were no other treatment-related histopathological abnormalities.

Conclusions

The NOAEL following 13-weeks of dietary exposure to Advantame was 50000 ppm

(equivalent to 4227 mg/kg bw/day in males and 5109 mg/kg bw/day in females), the highest concentration tested.

3.2.5.3 Dogs

Powell LAJ & Scott AM (2005) Toxicity study by dietary administration to beagle dogs for 13 weeks followed by a 4 week recovery period. Report No. AJO 179/014664. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. **GLP**: UK, EC, OECD, US FDA & Japan. **QA statement**: Yes. **Guidelines**: US FDA, EC & Japan ***** Baldrey SF, Flack I, McBurney A & Carr MJ (2005) Pharmacokinetics of ANS 9801 and ANS 9801-acid following dietary administration to beagle dogs for 13 weeks followed by a 4-week recovery period. Report No. AJO 179/014664.

Experimental

Advantame (99.5% purity; Batch No. 001227; sourced from the sponsor) was admixed in the diet and fed to 4 beagle dogs/sex/group at concentrations of 0, 5000, 15000 or 50000 ppm for 13 weeks. An additional two dogs/sex/group in the control and 50000 ppm groups were then maintained untreated for 4 weeks. The dietary concentrations were based on the results of a 4-week dietary study (Report No. AJO156/012585). Dogs were sourced from Ridglan Farms Inc (Mount Horeb, Wisconsin, USA) and at the commencement of dosing, were 23-26 weeks old. The bodyweight range was 7.4-10.8 kg in males and 6.1-8.3 kg in females. Dogs were housed 2/sex/kennel, with 400 g diet offered each day. Water was available *ad libitum*.

Observations for mortality and clinical signs were made at least twice daily, with a more detailed examination made at least weekly. Bodyweights were recorded weekly. Food consumption was recorded daily but reported on a weekly basis. Ophthalmoscopy was performed before treatment began and at wk 13. ECGs were recorded before treatment began, during wk 6 and 13 of treatment then during wk 4 of the recovery period. Standard haematology and clinical chemistry parameters (Appendix 1) were analysed in blood samples collected pre-treatment, wk 6 and 13 of the treatment period then during wk 4 of recovery. Water was withheld for 5 h prior to the collection of urine during these same times for the analysis of standard urinalysis parameters (Appendix 1).

For toxicokinetic analysis, blood was sampled on d 1 and 90 (pre-dose, 0.5, 1, 3, 6, 12 and 24 h) and d 8, 15, 22, 29 and 36 (pre-dose only); concentrations of Advantame and Advantame-acid were measured by LC-MS/MS. Standard toxicokinetic parameters were recorded/calculated. Dogs were killed after 13 week of treatment or following the 4-week recovery phase, then necropsied. Standard organs were weighed (plus the lungs and salivary gland) and histopathologically examined (plus the gall bladder and tongue) (Appendix 2).

Results

MORTALITIES & CLINICAL SIGNS

There were no deaths. Pale faeces were noted for all dogs at 50000 ppm, consistent with rodent studies, and are most likely attributable to the high concentration of test material in the GIT rather than to a direct toxicological effect. Loose/liquid stools occurred across all groups, with the incidence somewhat higher in treated dogs.

BODY WEIGHT & FOOD CONSUMPTION

At 50000 ppm, significantly reduced ($p \leq 0.05$) bodyweight gain (wk 0-13) occurred in males

(1.7±1.01, 1.0±0.68, 1.2±0.79 and 0.5±0.75 kg at 0, 5000, 15000 and 50000 ppm, respectively). However, the result is considered to reflect the relatively high concentration of a non-caloric substance (5%) in the diet of this group and that dogs were offered a fixed amount (400 g) of feed per day. In other words, this group received 5% less energy than the control group and therefore the finding is not attributable to a toxicological effect. Food consumption and female bodyweight gain was unremarkable.

The achieved doses were 205, 667 and 2230 mg/kg bw/day in males and 229, 703 and 2416 mg/kg bw/day in females at 5000, 15000 and 50000 ppm, respectively.

TOXICOKINETICS

Mean C_{max} and AUC_{24} values for Advantame and Advantame-acid at d 1 and 90 are presented in Table 3.21 but are viewed as estimates only due to the underestimation of the Advantame-acid concentration. Systemic exposure to Advantame-acid was higher than to Advantame as shown by the larger C_{max} and AUC_{24} values, with the large metabolite ratios indicating extensive metabolism to Advantame-acid. Based on the average metabolite ratio, it is estimated that a minimum of 97% of the administered dose of Advantame was converted to Advantame-acid prior to absorption. Given the underestimation of Advantame-acid due to the analytical uncertainty, the actual level of GIT conversion was probably closer to 100%.

TABLE 3.21: TOXICOKINETIC PARAMETERS IN DOGS

Parameter	5000 ppm		15000 ppm		50000 ppm	
	Males	***Females***	***Males***	***Females***	***Males***	***Females***
Advantame						
C_{max} (ng/mL)						
d 1	314 (1)	176 (1)	669 (2.1)	822 (4.7)	1687 (5.4)	2874 (16.3)
d 90	1209 (1)	2789 (1)	1594 (1.3)	2786 (1.0)	2184 (1.8)	4485 (1.6)
AUC_{24} (ng.h/mL)						
d 1	4243 (1)	3447 (1)	13010 (3.1)	15396 (4.5)	31975 (7.5)	51323 (14.9)
d 90	19318 (1)	48869 (1)	26519 (1.4)	42857 (0.9)	42843 (2.2)	81341 (1.7)
Advantame-acid						
C_{max} (ng/mL)						
d 1	11810 (1)	13302 (1)	28121 (2.4)	20388 (1.5)	72226 (6.1)	93974 (7.1)
d 90	45646 (1)	62767 (1)	57957 (1.3)	74813 (1.2)	94433 (2.1)	114906 (1.8)
AUC_{24} (ng.h/mL)						
d 1	207943 (1)	210446 (1)	462973 (2.2)	355670 (1.7)	1279722 (6.2)	1710599 (8.1)
d 90	788720 (1)	1251217 (1)	985703 (1.2)	1421269 (1.1)	1786376 (2.3)	1983731 (1.6)
Metabolite ratio[2]						
d 1	50.2	62.5	35.6	22.9	40.5	33.8
d 90	44.5	25.9	37.5	33.9	43.1	24.4

Results are expressed as the mean, with the C_{max} and AUC_{24} ratios contained in parentheses; 1 = AUC_{24} d 90 ÷ AUC_{24} d 1; 2 = AUC_{24} Advantame-acid ÷ AUC_{24} Advantame

OPTHALMOSCOPY & ECG

There were no treatment-related opthalmoscopic or ECG findings.

HAEMATOLOGY

There were no treatment-related haematological effects in males. Selected haematology findings in females are summarised in Table 3.22. There was no significant difference between treated and control groups during wk 6 of treatment. During wk 13 of treatment, significantly lower ($p<0.01$) Hct, Hb and RBC were determined at 15000 and 50000 ppm relative to the control group. At 50000 ppm, mean reticulocytes ($p<0.01$) and WBC ($p<0.05$) were also significantly lower than the control. The authors stated that the values fell within

the historical control range but no data were provided to substantiate this conclusion.

TABLE 3.22: SELECTED HAEMATOLOGY FINDINGS IN FEMALES

Parameter	0 ppm	5000 ppm	15000 ppm	50000 ppm
Hct (L/L)[1]				
Pretreatment	0.444±0.0095	0.455±0.0126	0.458±0.0169	0.437±0.0189
Wk 6	0.438±0.0197	0.428±0.0257	0.422±0.0263	0.421±0.024
Wk 13	0.447±0.0073	0.439±0.0153	0.412±0.0091**	0.408±0.0268**
Recovery	0.441	-	-	0.426
Hb (g/dL)[2]				
Pretreatment	14.8±0.26	14.8±0.38	14.7±0.49	14.2±0.58*
Wk 6	14.7±0.65	14.4±0.90	14.2±0.86	14.2±0.73
Wk 13	15.3±0.18	14.9±0.63	14.0±0.38**	13.7±0.92**
Recovery	15.6	-	-	14.8
RBC ($x10^{12}$/L)[3]				
Pretreatment	6.52±0.247	6.65±0.186	6.48±0.182	6.53±0.252
Wk 6	6.34±0.258	6.15±0.373	6.00±0.443	6.27±0.352
Wk 13	6.66±0.265	6.47±0.265	6.00±0.124**	6.19±0.220**
Recovery	6.51	-	-	6.37
Reticulocytes (%)				
Pretreatment	0.67±0.211	0.56±0.108	0.51±0.137	0.47±0.099*
Wk 6	0.76±0.228	0.63±0.147	0.80±0.404	0.68±0.182
Wk 13	0.82±0.269	0.53±0.152	0.64±0.125	0.44±0.123**
Recovery	0.65	-	-	0.48
WBC ($x10^9$/L)				
Pretreatment	14.00±2.072	11.25±2.273	10.73±4.128	10.65±1.757*
Wk 6	11.42±1.210	9.40±0.496	12.66±2.561	10.62±1.563
Wk 13	11.56±0.964	9.92±1.763	10.44±2.140	8.92±2.201*
Recovery	11.09	-	-	9.23

Results expressed as the mean ± 1 SD; *p≤0.05; **p≤0.01

1 = 0.41-0.55 L/L (95% range in young adult dogs) (Sibley et al 1974);
0.43±0.025 L/L (mean ± 1 SD in young adult dogs) (Aleman et al 2000)

2 = 13.6-19.4 g/dL (95% range in young adult dogs) (Sibley et al 1974)
14.86±1.31 g/dL (mean ± 1 SD in young adult dogs) (Aleman et al 2000)
15.27±1.18 g/dL (mean ± 1 SD in 12-month old females) [Ridglan Farms (date unspecified)]

3 = 5.9-8.1 10^{12}/L (95% range in young adult dogs) (Sibley et al 1974)
7.66±0.55 x 10^{12}/L (mean ± 1 SD for 12-month old female beagles) [Ridglan Farms (date unspecified)]

The significantly lower Hb, reticulocytes and WBC at 50000 ppm are not considered treatment-related as these parameters were already significantly lower than the control group prior to the commencement of dosing. With regard to Hct and RBC, the magnitude of the difference with the control group was relatively small and a dose-response relationship not evident. In the 15000 and 50000 ppm groups, mean Hct and RBC were lower than the respective pre-treatment values during wk 6 and 13. However, reductions in these parameters from pre-treatment to wk 6 occurred across all groups (and all dogs), including the control, and therefore the (non-significant) differences during wk 6 are not attributable to treatment. During wk 13, mean Hct and RBC increased from wk 6 in the control and 5000 ppm groups but tended to decrease by a further small magnitude in the 15000 and 50000 ppm groups. An examination of individual animal data indicated that the Hct and RBC of approximately half of the dogs in the 15000 and 50000 ppm groups decreased from wk 6 to wk 13 by a small magnitude, while slight increases were seen in the control and 5000 ppm groups. In the two 50000 ppm dogs allowed to recover for four weeks, only slight differences in Hct and RBC from wk 13 were noted (~2% decrease in one dog and ~2% increase in the other). In the two control dogs allowed to recover for four weeks, an increase in one dog and decrease in another (<5%) was noted. Collectively these findings suggest that the decreased Hct and RBC at 15000 and 50000 ppm are unlikely to be treatment-related and

reflect normal biological variation.

CLINICAL CHEMISTRY

There was no treatment-related effect on any clinical chemistry parameters in males. In 50000 ppm females, significant differences ($p<05$) to the control in a number of parameters were noted [higher cholesterol and phospholipids, lower α_2-globulin and higher β-globulin during wk 6; lower K during wk 6 and 13; lower creatinine during wk 13]. However, these statistical differences are not considered treatment-related on the basis of the following: numerically, K, α_2- and β-globulin changed little over 13 weeks of treatment relative to the pre-treatment values; relative to pre-treatment values, cholesterol and phospholipids actually decreased to wk 6 and 13; and all parameters remained unchanged from wk 13 to the end of the 4-week recovery period.

URINALYSIS

Urinalysis parameters were unremarkable.

ORGAN WEIGHTS, MACROPATHOLOGY & HISTOPATHOLOGY

Selected organ weights of dogs sacrificed after 13 weeks of treatment are presented in Table 3.23. Absolute pituitary weights were significantly elevated in males at 15000 and 50000 ppm ($p\leq0.05$ and 0.01, respectively) but in the absence of similar differences in females, the lack of an effect on relative pituitary weight and in the absence of any pathology of this organ, the differences are not considered treatment-related. The organ weights of dogs sacrificed after the 4 week recovery period were unremarkable.

The mean absolute and relative thymus weights of 50000 ppm males were approximately half that of the control group, a result that was not statistically significant. Corroborative evidence of a treatment-related effect included a macroscopically small thymus in 3/4 dogs in the same group (no control males had a small thymus) and histopathological evidence of involution/atrophy (1, 0, 1 and 2/4 dogs at 0, 5000, 15000 and 50000 ppm, respectively), which was graded as moderate at 15000 and 50000 ppm (and slight in the control group). Dogs sacrificed after the 4 week recovery period did not display these findings. The authors examined the relationship between thymic involution/atrophy, bodyweight gain and thymus weight and established that the occurrence of thymic involution/atrophy across all groups occurred in dogs that had the smallest bodyweight gain over 13 weeks and the smallest absolute thymus weight. A re-examination of the control groups from 5 studies conducted previously by the performing laboratory confirmed this relationship and indicated that the incidence and severity of effects on the thymus were consistent with these studies and therefore are not considered to be toxicologically significant.

TABLE 3.23: SELECTED ORGAN WEIGHTS (WEEK 13)

Organ	0 ppm		5000 ppm		15000 ppm		50000 ppm	
	Males	***Females***	***Males***	***Females***	***Males***	***Female***	***Males***	***Females***
Pituitary (absolute)	0.0775 (0%)	0.0605 (0%)	0.0728 **(+6%)**	0.0645 **(+7%)**	0.0613* **(+21%)**	0.0585 **(-3%)**	0.0590** **(+24%)**	0.0650 **(+7%)**
Pituitary (relative)[1]	0.00073 (0%)	0.00065 (0%)	0.00071 **(-3%)**	0.00074 **(+14%)**	0.00063 **(-14%)**	0.00066 **(+2%)**	0.00064 **(-12%)**	0.00078 **(+20%)**
Thymus (absolute)	9.05 (0%)	9.67 (0%)	8.37 **(-8%)**	7.71 **(-20%)**	7.63 **(-16%)**	9.09 **(-6%)**	4.47 **(-51%)**	7.30 **(-25%)**
Thymus (relative)	0.0856 (0%)	0.104 (0%)	0.0827 **(-3%)**	0.0889 **(-15%)**	0.0795 **(-7%)**	0.103 **(-1%)**	0.0491 **(-43%)**	0.0885 **(-15%)**

Absolute organ weights are expressed as the mean (g) $\pm$ 1 SD, with the % difference (+ or -) to the control given

in parentheses (bolded); relative organ weights are expressed as % of terminal bodyweight and were calculated by dividing the mean absolute organ weight by the mean terminal bodyweight then multiplying by 100; *p≤0.05; **p≤0.01; 1 = calculated by FSANZ

Conclusions

The NOAEL was 50000 ppm (equal to 2230 mg/kg bw/day in males and 2416 mg/kg bw/day in females) based on the absence of any toxicologically significant effect at this dietary concentration.

3.2.6 Chronic Toxicity & Carcinogenicity Studies

3.2.6.1 Mice

Horne C (2006a) ANS9801. Carcinogenicity study by dietary administration to CD-1 mice for 104 weeks. Report No. AJO 198/033050. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. **GLP**: UK, EC, OECD, US FDA & Japan. **QA statement**: Yes.

Experimental

This study was specifically designed to assess carcinogenicity rather than chronic toxicity and therefore only limited haematology parameters and no clinical chemistry or urinalysis parameters were analysed.

Advantame (99.3% purity; Batch No. 010228; sourced from the Sponsor) was admixed in the diet and fed *ad libitum* to groups of sixty-four Crl:CD-1 (ICR) BR mice/sex at concentrations of 0, 2000, 10000 or 50000 ppm for 104 weeks. These concentrations were chosen on the basis of a previous subchronic mouse study (Report No. 174/014348). Mice were sourced from Charles River UK Ltd (Margate, Kent), were 40-44 days old and weighed 26.2-45.2 g (males) or 20.9-30.3 g (females) at the commencement of dosing. They were housed 2/sex/cage, with water available *ad libitum*.

Observations for mortalities and clinical signs were made at least twice daily with more detailed examinations performed on a weekly basis. Bodyweight was recorded on the day dosing commenced and weekly thereafter. Food consumption was recorded weekly. Blood was collected from (fasted) surviving mice during wk 52 and 78 and smears examined from the control and 50000 ppm group; in the absence of any treatment effects at the high-dose, the mid- and low-dose groups were not examined. Blood was also collected during wk 104 of treatment for analysis of a limited number of haematology endpoints (RBC, WBC, differential WBC, abnormal morphology). All survivors were sacrificed after 104-weeks of treatment and necropsied. Any mice dying or killed *in extremis* during the study were also sacrificed. Standard organs were weighed and histopathologically examined (Appendix 2).

Results

MORTALITIES & CLINICAL SIGNS

There were no treatment-related deaths or clinical signs. Survival to 104 weeks was 31/41, 27/23, 34/33 and 38/27% in males/females at 0, 2000, 10000 and 50000 ppm, respectively. There was no difference in the incidence, multiplicity or time of onset of palpable swellings between treated and control groups.

BODYWEIGHT & FOOD CONSUMPTION

There was no treatment-related effect on absolute bodyweight. Bodyweight gain in males

was unaffected by treatment. In females, mean bodyweight gain to wk 16, 78 and 104 were lower than the control at 50000 ppm (10, 13 and 24% lower, respectively), with the overall bodyweight gain significantly lower ($p \leq 0.05$) than the control. There was no effect on food consumption. Food conversion efficiency was calculated to wk 16 only, which was unremarkable. The effect on female bodyweight gain is probably not attributable to a direct toxic effect of Advantame but to its relatively high dietary concentration (~5%) leading to reduced caloric intake over 2 years.

The mean achieved doses of Advantame over 104-weeks of treatment were 213/272, 1057/1343 and 5693/7351 mg/kg bw/day in males/females at 2000, 10000 and 50000 ppm, respectively.

HAEMATOLOGY

There were no treatment-related differences in blood smears evaluated at wk 52 and 78. In both sexes at 50000 ppm, mean RBC was significantly lower ($p \leq 0.01$ males; $p \leq 0.05$ females) than the respective control groups during wk 104 (8.48, 8.14, 7.92 and 7.27 x 10^{12}/L in males and 7.68, 7.30, 7.37 and 6.85 10^{12}/L in females at 0, 2000, 10000 and 50000 ppm, respectively). Considering that this study was specifically designed to assess the carcinogenicity of Advantame in a second laboratory animal species, and in the absence of any corroborative haematological data, the relevance of this finding is unclear.

ORGAN WEIGHTS

The mean terminal bodyweight of 50000 ppm females sacrificed after 104-weeks of treatment was significantly lower ($p<0.05$) than the control (39.3 *versus* 43.8 g, respectively). There was no significant difference in organ weights between treated and control mice, including those organs listed in Table 3.24 where relatively large elevations in mean absolute/relative weights occurred at 50000 ppm. Given the large variability in the results attributable to a small number of mice with anomalous organ weights at 50000 ppm [two males with thymus weights of 0.2020 (#196) and 0.1708 g (#232), and one female with a weight of 1.2819 g (#452); three mice with uterus/cervix weights of 7.682 (#453), 6.684 (#455) and 6.597 g (#476); one mouse with a left ovary weight of 8.0171 g (#476) and another with a right ovary weight of 6.2848 (#502)] the lack of a dose-response relationship and absence of any corroborative evidence from other endpoints, these differences are not considered treatment-related.

TABLE 3.24: ORGAN WEIGHT FINDINGS AFTER 104-WEEKS OF TREATMENT

Organ	0 ppm		2000 ppm		10000 ppm		50000 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
N	19	17	17	24	22	15	24	17
Thymus (absolute)	0.0189 ±0.0164	0.0628 ±0.0867	0.0151 ±0.0078 **(-20%)**	0.0722 ±0.0782 **(+13%)**	0.0138 ±0.0108 **(-27%)**	0.0555 ±0.0753 **(-11%)**	0.0321 ±0.0513 **(+41%)**	0.1119 ±0.3080 **(+44%)**
Thymus (relative)	0.0346	0.1487	0.0268 **(-24%)**	0.1843 **(+24%)**	0.0250 **(-26%)**	0.1106 **(-26%)**	0.0566 **(+63%)**	0.2718 **(+83%)**
Uterus & cervix (absolute)	-	1.430 ±1.893	-	1.304 ±1.289 **(-9%)**	-	1.104 ±1.104 **(-23%)**	-	2.134 ±2.571 **(+49%)**
Uterus & cervix (relative)	-	3.4465	-	3.4279 **(0%)**	-	2.6864 **(-22%)**	-	5.3972 **(+57%)**
Ovaries (absolute)	-	1.4090 ±2.1565	-	1.1125 ±1.1035 **(-21%)**	-	1.3495 ±2.2658 **(-4%)**	-	2.6469 ±2.7652 **(+88%)**

Organ	0 ppm		2000 ppm		10000 ppm		50000 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
Ovaries (relative)		3.2610		2.8815 **(-12%)**		2.7745 **(-15%)**		6.5813 **(+100%)**

Absolute organ weights expressed as the mean (g) ± 1 SD, with the % difference (+ or -) to the control given in parentheses (bolded). Relative organ weights are expressed as % of terminal bodyweight.

MACROPATHOLOGY

Selected macroscopic findings for all mice (those found dead, killed *in extremis* or killed after 104-weeks of treatment) are summarised in Table 3.25. The incidence of exophthalmos and harderian gland masses were increased in males across all treated groups relative to the control. These findings were concomitant with an increased incidence of adenoma of the Harderian glands (Table 3.26). However, given the lack of a dose-response relationship, a similar increase in females and that the incidence of adenoma was within the historical control range for CD-1 Mice [1.67-18.64%; CRL (2005)], these findings are not considered treatment-related.

In males, the appearance of an enlarged liver was increased across all treatment groups relative to the control, reaching statistical significance at 50000 ppm ($p<0.05$). However, as there was no dose-response relationship, female livers appeared normal and as absolute and relative liver weights were unremarkable, it was not considered treatment-related.

TABLE 3.25: MACROSCOPIC FINDINGS

Organ	0 ppm		2000 ppm		10000 ppm		50000 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
N	64	64	64	64	64	64	64	64
Exophthalmos	1 (1.5%)	7 (11%)	3 (4.7%)	6 (9.4%)	4 (6.3%)	5 (7.8%)	5 (7.8%)	4 (6.3%)
Enlarged liver	0 (0%)	8 (12.5%)	5 (7.8%)	5 (7.8%)	4 (6.3%)	7 (11%)	6* (9.4%)	5 (7.8%)
Harderian gland masses	1 (1.6)	3 (4.7%)	5 (7.8%)	2 (3.1%)	5 (7.8%)	1 (1.6%)	6 (9.4%)	2 (3.1%)

Results expressed as the absolute number of mice showing the abnormality, with the % incidence contained in parentheses; * $p<0.05$

HISTOPATHOLOGY

Neoplastic findings: Selected neoplastic findings are summarised in Table 3.26. The incidence of haemangiosarcoma of the liver (males) and bronchioloalveolar adenocarcinoma (females) was increased across all treated groups relative to the control. However, in the absence of a dose-response relationship, a similar increase in the opposite sex and that all values fell within the historical control ranges for sex-matched CD-1 mice between 78-104-weeks old, the findings are not considered treatment-related.

TABLE 3.26: NEOPLASTIC FINDINGS

Organ	0 ppm		2000 ppm		10000 ppm		50000 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
Haemangiosarcoma (liver)[1]	0/63 (0%)	0/63 (0%)	3/63 (4.8%)	1/63 (1.6%)	4/64 (6.3%)	1/63 (1.6%)	2/63 (3.2%)	0/64 (0%)
Bronchioloalveolar adenocarcinoma	11/64 (17%)	1/64 (1.7%)	14/64 (2.2%)	7/64* (11%)	6/64 (9.4%)	5/63 (7.9%)	6/64 (9.4%)	5/64 (7.8%)
Adenoma (Harderian glands)	1/64 (1.6%)	2/64 (3.1%)	2/64 (3.1%)	1/64 (1.6%)	3/64 (4.7%)	1/64 (1.6%)	6/64 (9.4%)	1/64 (1.6%)

Results expressed as the absolute number of mice showing the abnormality, with the % incidence contained in parentheses; * $p<0.05$

1 = historical control range in males: 1.11-8.57% (CRL 2005)

2 = historical control range in females: 0.77-26% (CRL 2005)

Non-neoplastic findings: There were no treatment-related non-neoplastic findings.

Conclusions

The NOAEL for carcinogenicity was 50000 ppm (equal to 5693 mg/kg bw/day in males and 7351 mg/kg bw/day in females), the highest dietary concentration tested.

3.2.6.2 Rats

Horne C et al (2005) ANS9801: Combined carcinogenicity and toxicity study by dietary administration to Han Wistar rats for 104 weeks with an *in utero* exposure phase. Interim Report. Report No. AJO 195/033047. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. **GLP**: UK, OECD, EC, Japan, US FDA. **QA statement**: Yes.

Horne C et al (2006b) ANS9801: Combined carcinogenicity and toxicity study by dietary administration to Han Wistar rats for 104 weeks with an *in utero* exposure phase. Report No. AJO 195/033048. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. **GLP**: UK, OECD, EC, Japan, US FDA. **QA statement**: Yes.

Holding JD (2005a) Interim bioanalytical report. ANS9801: Combined carcinogenicity and toxicity study by dietary administration to Han wistar rats for 104 weeks with an *in utero* exposure phase. Report No. AJO 195/033047. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan.

Holding JD (2005b) Toxicokinetic report. ANS9801: Combined carcinogenicity and toxicity study by dietary administration to Han wistar rats for 104 weeks with an *in utero* exposure phase. Report No. AJO 195/033048. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan.

Experimental

Advantame (≥99.2% purity; Lot No. 010212; sourced from the Sponsor) was admixed in the diet at concentrations of 0, 2000, 10000 or 50000 ppm and fed *ad libitum* to groups of 55 Hsd Brl Han:Wist rats per sex/group for 104 weeks (sourced from Harlan UK Ltd, Oxfordshire, England) from approximately 4-weeks of age. The dose selection was based on the results of a previous 13-week dietary study. Rats were the offspring of parents that had been exposed to the same dietary concentrations of Advantame from 4 weeks before mating to weaning. Twenty additional rats/sex were allocated to each treatment group for interim sacrifice at wk 52. Another 10 rats/sex were assigned to the control, 10000 and 50000 ppm groups and allowed to recover for 6 weeks from wk 52, prior to sacrifice. Satellite groups of 12 rats/sex were treated for 104-weeks and their blood sampled at wk 14, 26, 52 and 104 for the analysis of Advantame and Advantame-acid by LC-MS/MS. Rats were housed individually under standard conditions, with water available *ad libitum* except prior to urine collection or blood sampling when it was withdrawn overnight.

Observations for clinical signs were made at least twice daily, with a more comprehensive examination performed weekly. Bodyweight and food consumption were recorded weekly. Water consumption was recorded during wk 11, 24 and 50 of treatment (interim sacrifice phase), and wk 4 of the recovery period. Ophthalmoscopy was performed on all rats assigned to the interim sacrifice and recovery phases during wk 1, and then during wk 13, 26, 39 and 52 for the control and high-dose groups.

Blood was collected from 10 fasted rats/sex/group during wk 13, 26, 39 and 52, and wk 6 of

recovery in rats assigned to the recovery phase. Rats assigned to the main study were also sampled during wk 78 and 104. Standard haematology and clinical chemistry parameters were analysed (Appendix 1). Overnight urine was collected during wk 12, 25, 38 and 51 from rats assigned for interim sacrifice, during wk 51 of treatment and wk 5 of recovery from rats assigned to the recovery phase, and during wk 77 and 103 from rats assigned to the main study; standard urinalysis parameters were analysed (Appendix 1). Following sacrifice, rats were necropsied and their organs weighed. Standard organs/tissues were histopathologically examined (Appendix 2).

Results

REPRODUCTION AND *IN UTERO* EXPOSURE PHASE

There were no treatment-related mortalities or clinical signs in parental rats.

During the 4-week pre-mating period, wk 1 food consumption of males was significantly lower ($p<0.05$ or 0.01) than the control at 10000 and 50000 ppm (150 and 146 g, respectively, *versus* 156 g in the control) but thereafter was higher than the control group. In contrast, food consumption was significantly higher ($p<0.01$) in 50000 ppm females during wk 2 and 3 (~9.5% higher than the control). At every dietary concentration, the bodyweight gain of dams was significantly higher ($p<0.01$) than the control from d 1-14 of lactation (30, 35, 36 and 40 g at 0, 2000, 10000 and 50000 ppm, respectively). This increase was concomitant with increased food consumption, which was significantly higher ($p<0.01$ or 0.05) than the control during d 7-13 of lactation (52, 55, 57 and 58 g at 0, 2000, 10000 and 50000 ppm, respectively). None of these findings are considered adverse.

There were no treatment-related, adverse effects on mating or gestation length, litter sizes, sex ratios, or on the growth and survival of offspring.

MORTALITIES & CLINICAL SIGNS

At 50000 ppm, up to 52/85 and 62/85 males and females, respectively, had a swollen anus, which had resolved in the majority of affected rats by wk 32. Pallor of the anus was observed in a smaller number of rats at this same dietary concentration (up to 29/85 and 14/85 males and females, respectively). Only two males displayed these signs at 10000 ppm. Discolouration of the faeces (light, dark, green or striped) and green staining of the cage lining (attributed by the authors to contact with faeces) occurred at 10000 and 50000 ppm in both sexes, declining over the duration of dosing but remaining evident to wk 104. The authors attributed the light coloured faeces in the majority of 50000 ppm rats (up to 85/85 and 83/84 males and females, respectively) to the high concentration of Advantame in the diet, while the occurrence of dark or green faeces (up to 23/85 and 35/84 males and females, respectively) was attributed to Advantame-acid. While these findings are clearly treatment-related they are not considered toxicologically significant.

During the main study, palpable swellings were observed across all groups, with their incidence and time of onset showing no relationship to treatment.

BODYWEIGHT GAIN, FOOD & WATER CONSUMPTION

At 50000 ppm, bodyweight gain to week 52 in males was significantly lower ($p<0.05$) than the control (95% of the control). Over the 6-week recovery phase, this same group had a significantly higher ($p<0.01$) bodyweight gain than the control (162% of the control). Mean bodyweight gain over the 104 weeks of the main study was lower than the control at 50000 ppm in males and females (~93% of the control), but was statistically significant ($p<0.05$) only in males. There was no treatment-related effect on food consumption or food

conversion efficiency.

Mean water consumption was significantly higher ($p<0.01$ or 0.05) than the control across all treated groups during wk 11 (121/118, 114/111 and 117/118% higher than the control in males/females at 2000, 10000 and 50000 ppm, respectively). At later sampling intervals, mean water consumption of treated rats was marginally higher than the control reaching statistical significance ($p<0.05$) in males at 10000 and 50000 ppm during wk 24 (104 and 107% of the control, respectively), and at 2000 ppm in females during wk 50 (118% of the control; $p<0.05$). In the absence of a dose-response relationship or other evidence of a direct toxicological effect, these treatment-related effects on water consumption are not considered toxicologically significant; the authors suggested the findings may have been due to the taste of the test material.

Mean achieved doses over the study were 120/149, 610/743 and 3279/4025 mg/kg bw/day in males/females at 2000, 10000 and 50000 ppm, respectively.

TOXICOKINETICS

Advantame and Advantame-acid were detectable in all samples. Mean C_{max} and AUC_{24} values for Advantame and Advantame-acid are presented in Table 3.27. Systemic exposure to Advantame-acid was higher than exposure to Advantame as shown by the larger C_{max} and AUC_{24} values. C_{max} values for Advantame and Advantame-acid were variable. For Advantame-acid, C_{max} and AUC_{24} values generally increased over the dose range in a non-linear manner. There was no consistent sex- or time-related difference in AUC_{24} for Advantame or Advantame-acid. Based on the average metabolite ratio (calculated by FSANZ), it is estimated that a minimum of 90% of the administered dose of Advantame was converted to Advantame-acid prior to absorption.

TABLE 3.27: C_{MAX} AND AUC_{24} VALUES FOR ADVANTAME AND ADVANTAME-ACID.

Parameter	2000		10000		50000	
	Males	***Females***	***Males***	***Females***	***Males***	***Females***
Advantame						
C_{max} (ng/mL)						
wk 14	46.8 (1)	86.6 (1)	20.8 (0.4)	56.0 (0.6)	97.5 (2.1)	92.8 (1.1)
wk 26	135 (1)	299 (1)	341 (2.5)	166 (0.6)	99.7 (0.7)	154 (0.5)
wk 52	61.2 (1)	45.3 (1)	68.5 (1.1	105 (2.3)	61.1 (1.0)	242 (5.3)
wk 104	29.7 (1)	33.2 (1)	118 (4.0)	70.5 (2.1)	194 (6.5)	332 (10.0)
AUC_{24} (ng.h/mL)						
wk 14	595 (1)	813 (1)	393 (0.7)	840 (1.0)	1660 (2.8)	1720 (2.1)
wk 26	1020 (1)	1560 (1)	1880 (1.8)	1630 (1.0)	1520 (1.5)	1730 (1.1)
wk 52	661 (1)	509 (1)	731 (1.1)	1180 (2.3)	934 (1.4)	1900 (3.7)
wk 104	426 (1)	490 (1)	1140 (2.7)	981 (2.0)	1510 (3.5)	3490 (7.1)
Advantame-acid						
C_{max} (ng/mL)						
wk 14	142 (1)	245 (1)	537 (3.8)	378 (1.5)	1710 (12.0)	2910 (11.9)
wk 26	128 (1)	216 (1)	661 (5.2)	658 (3.0)	2500 (19.5)	4450 (20.6)
wk 52	84.4 (1)	89.8 (1)	448 (5.3)	468 (5.2)	959 (11.4)	3310 (36.9)
wk 104	130 (1)	64.2 (1)	321 (2.5)	197 (3.1)	513 (3.9)	1880 (29.3)
AUC_{24} (ng.h/mL)						
wk 14	2310 (1)	3010 (1)	6950 (3.0)	7030 (2.3)	22800 (9.9)	44700 (14.9)
wk 26	2140 (1)	2770 (1)	10500 (4.9)	8530 (3.1)	25100 (11.7)	59800 (21.6)
wk 52	1450 (1)	1720 (1)	6380 (4.3)	6530 (3.8)	14400 (9.9)	42600 (24.8)
wk 104	1570 (1)	1110 (1)	5270 (3.4)	3910 (3.5)	7460 (4.8)	25700 (23.2)
Metabolite ratio[1]						

Parameter	2000		10000		50000	
	Males	***Females***	***Males***	***Females***	***Males***	***Females***
wk 14	3.9	3.7	17.7	8.4	13.7	26.0
wk 26	2.1	1.8	5.6	5.2	16.5	34.6
wk 52	2.2	3.4	8.7	5.5	15.4	22.4
wk 104	3.7	2.3	4.6	4.0	4.9	7.4

Results expressed as the mean, with the C_{max} and AUC_{24} ratios contained in parentheses; 1 = AUC_{24} for Advantame-acid ÷ AUC_{24} for Advantame

HAEMATOLOGY

At 50000 ppm in the interim sacrifice study, significantly lower ($p<0.05$ or 0.01) large unstained cells (LUC) occurred in males during wk 13 (0.05 *versus* 0.10 x 10^9/L in the control), 26 (0.06 *versus* 0.09 x 10^9/L in the control), 39 (0.08 *versus* 0.10 x 10^9/L in the control) and 52 (0.09 *versus* 0.13 x 10^9/L in the control) but not recovery wk 6. During week 26, significantly reduced LUC also occurred at 2000 and 10000 ppm (0.06 x 10^9/L at both doses *versus* 0.09 x 10^9/L in the control). In males in the main study, there was no significant difference in LUC during wk 78, while during wk 104, LUC was significantly lower ($p<0.5$) than the control (0.20 *versus* 0.29 x 10^9/L, respectively). As the majority of these values fell within the historical control range of LUC in male Han Wistar rats aged 17-weeks or older (0-0.07 x 10^9/L; CRL 2008), they are not considered abnormal. Indeed, these significance differences are a likely result of the abnormally high number of LUC in the control group. On this basis and in the absence of a dose-response relationship, and that a decrease in LUC is clinically questionable, these differences are not considered to reflect a treatment-related or toxicologically significant effect.

At 50000 ppm, monocytes were significantly lower ($p<0.05$) than the control in males during wk 13 (0.13 *versus* 0.22 x 10^9/L, respectively) and in females during wk 39 (0.12 *versus* 0.17 x 10^9/, respectively; $p<0.05$) and 52 (0.13 *versus* 0.18 x 10^9/L, respectively; $p<0.05$). During wk 78, monocytes were significantly lower in males across all doses (0.28, 0.22, 0.22 and 0.21 x 10^9/L at 0, 2000, 10000 and 50000 ppm, respectively). In the absence of a dose-response relationship and as all these values fell within the normal control range for Han Wistar rats >17-weeks of age (0.03-0.27 x 10^9/L males and 0.01-0.13 x 10^9/L in females; CRL 2008), they are not considered treatment-related.

In the 13-week study rat study by Chase et al (2004), Hct, Hb and lymphocytes were significantly lower than the control at 15000 and 50000 ppm during wk 13 relative to the control group. In the current study, no differences were noted in Hct, Hb, RBC, lymphocytes or any other haematology parameters during wk 13, 26, 39, 52, 78 or 104.

CLINICAL CHEMISTRY

Statistical analysis of clinical chemistry parameters determined a number of significant differences between treated and control groups, which were considered to be incidental in nature based on the lack of a dose-response relationship, the lack of consistency over time or between sexes, or that the direction of the change (increase or decrease) was not biologically relevant. However, there are several findings worth some discussion. Firstly, P was significantly higher ($p<0.01$) than the control at 50000 ppm in both sexes during wk 13 (males: 2.25 *versus* 1.96 mmol/L, respectively; females: 1.98 *versus* 1.62 mmol/L, respectively). The historical control range for P in Han Wistar rats aged greater than 17-week old is 1.18-2.71 mmol/L in males and 1.49-3.09 mmol/L in females (CRL 2008) and therefore these mean values are not abnormal. Further, the absence of a significant difference at any other sampling interval suggests that this finding is not treatment-related.

In males, plasma Ca was significantly lower ($p<0.01$) than the control during wk 13 at every

dose (2.92, 2.85, 2.80 and 2.79 mmol/L at 0, 2000, 10000 and 50000 ppm, respectively). However, given that it was significantly *increased* ($p<0.01$) at 50000 ppm during week 26 in males (2.77 *versus* 2.70 mmol/L in the control) and females (2.96 *versus* 2.82 mmol/L in the control), there were no differences at other sampling intervals (week 39, 52, 78 and 104) and as all values fell within the historical control range for Han Wistar rats >17-weeks of age [2.275-2.95 and 2.375-3.025 mmol/L in males and females, respectively; CRL (2008)], this finding is not considered treatment-related.

Plasma urea concentrations are summarised in Table 3.28 and indicate significantly lower ($p<0.01$ or 0.05) concentrations at 50000 ppm at all sampling weeks with the exception of the recovery phase. An examination of individual animal data revealed that the lowest values at 50000 ppm fell outside the concurrent control range. The lower plasma urea concentrations may reflect the reduced intake of protein due to the presence of Advantame in the diet and is consistent with results of the 13-week study (Chase et al 2004). On this basis, the lower plasma urea levels are considered to be a treatment-related adaptive response and hence not adverse. An adaptive response is supported by the observation that the urea values fell within the normal reference range for Han Wistar rats >17-weeks old [3.82-7.14 mmol/L in males and 4.18-8.92 mmol/L in females; (CRL 2008)].

TABLE 3.28: MEAN PLASMA UREA CONCENTRATIONS (MMOL/L)

Week	0 ppm		2000 ppm		10000 ppm		50000 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
13	7.54	7.55	7.63	7.93	7.79	8.10	6.78	6.77
26	6.86	8.27	6.26	7.69	6.42	7.16	6.15*	6.26**
26	6.69	7.06	5.98*	6.89	6.00*	7.28	5.53**	6.11*
52	5.92	6.65	5.58	7.07	5.82	6.51	5.29**	6.29
Recover wk 6	5.59	5.83	-	-	5.71	6.50	5.72	6.02
wk 78	5.22	6.65	5.01	5.22*	5.56	5.69*	4.41*	5.54*
wk 104	4.66	4.95	4.87	5.85	4.82	6.01	4.19*	5.40

*p<0.05; **p<0.01

At wk 78, the mean CPK concentration was significantly elevated ($p<0.05$) in males at 50000 ppm relative to the control (503$\pm$848.5 *versus* 158$\pm$95.7 U/L, respectively). However, this results is considered anomalous because it occurred at no other sampling time, was absent in females and was clearly attributable to a single outlying rat with very high CPK (2726 U/L).

Serum creatinine was significantly lower ($p<0.05$) in 50000 ppm males during wk 78 (44 versus 49 µmol/L in the control) and in females at every dietary concentration during wk 104 (49 µmol/L at every dietary concentration *versus* 53 µmol/L in the control). Given that decreased creatinine is not normally considered toxicologically relevant, and in the absence of similar differences at earlier sampling intervals or a dose-response relationship, these observations are not considered treatment-related.

URINALYSIS

Statistical analysis of urinalysis parameters determined a number of significant differences between treated and control groups, which were considered to be incidental in nature based on the lack of a dose-response relationship and the lack of consistency over time or between sexes.

ORGAN WEIGHTS

In males killed after 52 weeks of treatment, mean absolute and relative pituitary weights at 50000 ppm were significantly higher than the control group [0.011 *versus* 0.009 g, respectively ($p<0.05$); 0.0022 *versus* 0.0018, respectively ($p<0.01$)], which equates to an

approximately 20% difference. These differences were not evident in rats killed after 6 weeks of recovery. In the absence of a similar observation in rats killed after 104-weeks of treatment, a dose-response relationship, similar differences in females and macroscopic or histopathological abnormalities of this organ, these findings are not considered treatment-related. In males killed after 104 weeks of treatment, the mean absolute and relative weight of the seminal vesicles was approximately 17% and 23% higher, respectively, than the control at 50000 ppm ($p<0.05$ and 0.01, respectively) but in the absence of a dose-response relationship, or any histopathology of this tissue, the finding is not considered treatment-related.

In females sacrificed after 52 weeks of treatment, the mean absolute weight of the uterus and cervix was lower than the control at 10000 and 50000 ppm (0.735, 0.770, 0.660 and 0.615 g at 0, 2000, 10000 and 50000 ppm, respectively), but was statistically significant ($p<0.05$) only at 50000 ppm. These differences were not evident in rats killed after 6 weeks of recovery. In rats killed after 104 weeks of treatment, mean absolute and relative uterus and cervix weights were approximately 20% lower than the control at 50000 ppm, but not statistically significant. In the absence of any treatment-related histopathology of this organ, these findings are not considered treatment-related.

In rats sacrificed after 104 weeks of treatment, mean absolute and relative salivary gland weights were increased at 50000 ppm relative to the control (4/10% and 8/14% increases, respectively, in males/females), with the difference statistically significant for the absolute weight in females ($p<0.05$) and for the relative weight in both sexes ($p<0.01$). Concomitantly, there was an increase in the incidence of focal basophilic hypertrophy of the salivary glands in females at 10000 and 50000 ppm (Table 33), which was not significantly different to the control. Given the relatively modest magnitude of the differences in salivary gland weight, that there was no increase in the severity of basophilic hypertrophy (it was graded as minimum focal or multifocal across all groups, including the control) and given that it is a known age-related finding in rats (Whiteley et al 1996), these findings are not considered treatment-related.

MACROPATHOLOGY

In rats sacrificed after 52 weeks of treatment, the incidence of dark areas on the adrenals was increased in 50000 ppm females relative to the control [4/20 (20%) *versus* 1/19 (5%), respectively). The incidence in males was slightly higher than the control at 10000 and 50000 ppm [2/19 (10%) and 1/20 (5%), respectively, *versus* 0/20 (0%) in the control]. The incidence of this finding remained higher than the control following the 6-week recovery period [0, 3/10 (30%) and 2/10 (20%) in females and 0/10, 1/10 (10%) and 1/10 (10%) in males at 0, 10000 and 50000 ppm, respectively). In males sacrificed after 104-weeks of treatment, the incidence of pale areas of the adrenals was significantly increased ($p<0.05$) at 50000 ppm [19/38 (50%), 18/38 (47%), 20/30 (51%) and 30/39 (77%) at 0, 2000, 10000 and 50000 ppm, respectively)]. In the absence of any differences in the weight of the adrenals between treated and control groups, or any histopathology, these findings are considered incidental in nature.

In female decedants, abnormal GIT contents were observed across all treated groups [0/19 (0%), 7/22 (32%), 5/25 (20%) and 12/26 (42%) at 0, 2000, 10000 and 50000 ppm, respectively)], with the incidence at 2000 and 50000 ppm significantly different ($p<0.01$) to the control. A similar increase was not observed in male decedents or in survivors of either sex. An examination of individual animal data revealed the same abnormal contents across all groups of male decedents (yellow viscous fluid or dark contents in the lower GIT), which were consistent with most of the findings in females; on this basis the findings in females are unlikely to be treatment-related. Green stomach contents (5 rats) and ileum contents (1 rat) were observed uniquely in high-dose female decedants. None of these findings are

considered adverse.

In females sacrificed after 104 weeks of treatment, there was a dose-related increase in the incidence of “regional to mass” in the axillary lymph node [1/36 (2.7), 2/33 (6%), 4/30 (13%) and 6/29 (21%) at 0, 2000, 10000 and 50000 ppm, respectively), which was significantly different ($p<0.05$) to the control at the highest dose. In the absence of any histopathology, a similar finding in males or in the 6 other types of lymph nodes examined, the finding is not considered treatment-related.

HISTOPATHOLOGY

Neoplastic findings: There were no treatment-related histopathological abnormalities in rats designated for interim sacrifice at 52 weeks or in rats dying or killed during the 104-week treatment period.

In rats sacrificed after the 104-week treatment period, the incidence of benign mammary adenoma was increased across all treatment groups relative to the control [2/17 (12%), 4/20 (20%), 4/21 (19%) and 4/21 (19%) at 0, 2000, 10000 and 50000 ppm, respectively]. The trend test was statistically significant ($p=0.002$), while pair-wise comparisons revealed a significant difference ($p>0.036$) only between the 50000 ppm and control group. When the incidence of benign mammary adenomas and fibroadenomas were combined, there was a significant ($p=0.026$) dose-related increase [6/43 (14%), 8/44 (18%), 9/42 (21%) and 12/41 (29%) at 0, 2000, 10000 and 50000 ppm respectively)], which was not evident when the high-dose group was excluded from the analysis. Pair-wise comparisons revealed a significant difference ($p=0.042$) only between the 50000 ppm and control groups. As the combined incidence of mammary adenomas and fibroadenomas was within the historical control range for female Han Wistar rats aged 104-weeks [12.7-37.5%; CRL (2003)], these findings are not attributable to treatment.

The pooled incidence of pancreatic islet cell carcinoma was increased in males [0/55, 1/55 (1.8%), 2/55 (3.6%) and 3/55 (5.5%) at 0, 2000, 10000 and 50000 ppm, respectively] but not significantly. The historical control range for islet cell carcinoma in male Han Wistar rats is 0-10% (Tennekes et al 2004) and therefore this finding is considered to be within the range of normal biological variation and does not reflect a treatment-related effect.

Non-neoplastic findings: Selected histopathology findings in rats sacrificed after 52 or 104 week of treatment are presented in Table 3.29. In rats sacrificed after 52-weeks of treatment, pelvic dilatation of the kidneys was observed in males (graded as minimal/slight focal) at every dietary concentration, with the incidence at 50000 ppm significantly different ($p<0.05$) to the control. In females, there was a slight increase in proliferation of the urothelium of the kidneys at 50000 ppm relative to the control. In the absence of any evidence of kidney dysfunction, the lack of consistency between males and females, that there was no dose-related increase in the severity of these findings, and that similar findings were not evident in rats sacrificed after 104-weeks of treatment, neither finding is considered treatment-related.

TABLE 3.29: SELECTED HISTOPATHOLOGY FINDINGS AFTER 52 OR 104 WEEKS OF TREATMENT

Finding	0 ppm		2000 ppm		10000 ppm		50000 ppm	
	♂	♀	♂	♀	♂	♀	♂	♀
52 weeks								
N	20	19	20	20	19	20	20	20
Pelvic dilatation (kidneys)	0 (0%)	1 (5%)	3 (15%)	1 (10%)	2 (11%)	3 (15%)	6* (30%)	0 (0%)
Proliferation of urothelium (kidneys)	0 (0%)	1 (5%)	1 (5%)	0 (0%)	0 (0%)	2 (10%)	0 (0%)	3 (15%)

Finding	0 ppm		2000 ppm		10000 ppm		50000 ppm	
	♂	♀	♂	♀	♂	♀	♂	♀
Dilated/cystic sinuses (mesenteric lymph node)	2 (10%)	0 (0%)	3 (15%)	2 (10%)	5 (26%)	1 (5%)	6 (30%)	1 (5%)
Vacuolation of epithelium (prostate)	0 (0%)	-	2 (10%)	-	2 (11%)	-	3 (15%)	-
Focal basophilic hypertrophy (salivary glands)	0 (0%)	3 (16%)	0 (0%)	2 (10%)	0 (0%)	6 (30%)	0 (0%)	7 (35%)
104 weeks								
N	38	36	38	33	39	30	39	29
Sinusoidal dilatation/congestion (adrenals)	18 (47%)	36 (100%)	15 (39%)	32 (97%)	20 (51%)	26* (87%)	28* (72%)	27 (93%)
Extracapsular hyperplasia (adrenals)	1 (2.6%)	0 (0%)	0 (0%)	3 (9%)	0 (0%)	1 (3.3%)	4 (10.2%)	3 (10.3%)
Dilated tubules (kidneys)	30 (79%)	36 (100%)	37* (97%)	23** (70%)	36 (92%)	27 (90%)	37* (95%)	26 (90%)
Inflammatory cells (bladder)	0 (0%)	0 (0%)	2 (5.3%)	4* (12.5%)	1 (2.6%)	1 (3.3%)	3 (7.7%)	0 (0%)
Sinus erythrocytosis/ erythrophagocytosis (renal lymph node)	1 (2.6%)	0 (0%)	2 (5.3%)	0 (0%)	2 (5.1%)	1 (3.3%)	5 (13%)	1 (3.4%)

Results are expressed as the absolute number of rats affected, with the % incidence contained in parentheses;*p<0.05; **p<0.01

In males sacrificed after 52-weeks of treatment, the incidence of dilated or cystic sinuses of the mesenteric lymph node was increased in treated males but was not significantly different to the control group (10, 15%, 26 and 30% at 0, 2000, 10000 and 50000 ppm, respectively). As this findings is a common age-related, non-proliferative lesion in rats (Frith et al 2000), it is not considered attributable to treatment. In rats sacrificed after 104-weeks of treatment, the incidence of erythrocytosis/erythrophagocytosis of the sinus of the renal lymph node was increased relative to the control, but not significantly (2.6, 5.3, 5.1 and 13% in males and 0, 0, 3.3 and 3.4% in females at 0, 2000, 10000 and 50000 ppm, respectively). This particular finding can be an artefact of the tissue processing procedure (Elmore 2006) and therefore on its own is unlikely to represent a treatment-related abnormality.

In males sacrificed after 52-weeks of treatment, vacuolation of the epithelium of the prostate gland was slightly increased across all treatment groups relative to the control. In the absence of any abnormality of the prostate, that the finding was consistently graded as minimal multifocal across all treatment groups and that no treatment-related abnormalities of the prostate were noted in rats sacrificed after 104-weeks, it is not considered treatment-related.

In males sacrificed after 104-weeks of treatment, the incidence of sinusoidal dilatation or congestion of the adrenals was increased at 10000 and 50000 ppm, reaching statistical significance at 50000 ppm ($p<0.05$). This contrasts with the incidence in females, which was *lower* than the control at every dietary concentration, reaching statistical significance at 10000 ppm ($p<0.05$). On the basis of this inconsistent result, it is not considered to be treatment-related. Extracapsular hyperplasia of the adrenals was increased in males at 50000 ppm and in females at every dietary concentration, but not significantly. In the absence of a dose-response relationship, this finding is not considered toxicologically-significant.

Significant differences in the incidences of dilated kidney tubules and inflammatory cells of the bladder between treated and control groups were not considered treatment-related based on the absence of a dose-response relationship and the inconsistent results between males and females.

Conclusions

The NOAEL was 50000 ppm (equal to 3199 mg/kg bw/day in males and 4009 mg/kg bw/day in females) based on the absence of any toxicologically-significant effects at the highest dietary concentration tested.

3.2.6.3 Dogs

Powell LAJ, Lelasseux M-C & Crome SJ (2005) ANS9801: Toxicity study by oral dietary administration to beagle dogs for 52 weeks followed by a 6 week recovery period. Report No. AJO 196/034055. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. **GLP**: UK, OECD, EC, Japan, US FDA. **QA statement**: Yes. **Guidelines**: US FDA, EC, Japan.

Holding JD (2004) Toxicokinetic Report. ANS9801: Toxicity study by dietary administration to beagle dogs for 52 weeks followed by a six week recovery period. Report No. AJO 196/034055. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan.

Experimental

Advantame (>97% purity; Batch Nos. 010319 & 010626; sourced from the Sponsor) was admixed in the diet at concentrations of 0, 2000, 10000 or 50000 ppm and fed to groups of 4 beagle dogs/sex for 52 weeks. An additional two dogs/sex were assigned to the control, 10000 ppm and 50000 ppm groups and maintained untreated for a further 6 weeks. The dose selection was based on the results of a previous 13-week study (Report No. 179/014664). Dogs were sourced from Ridglan Farms Inc (WI, USA). Dogs were approximately 22-26 weeks of age, with bodyweights ranging from 7.6-10.0 kg in males and 6.4-9.0 kg in females. Dogs were housed under standard conditions, with water available *ad libitum*. Up until the end of wk 14 of treatment, dogs were offered 400 g of pelleted diet/day. However, from wk 15, dogs were offered 500 g of diet moistened with 750 g of water because of their lean appearance. From wk 34, one high-dose female (#414) was offered an additional 100 g of untreated pelleted diet in the afternoon following consumption of the treated diet.

Observations for mortality and clinical signs were made at least twice daily, with a more detailed examination made at least weekly. Bodyweights were recorded at least weekly prior to feeding. Food consumption was recorded daily but reported on a weekly basis. Ophthalmic examinations were performed before treatment began and during wk 12, 25, 38 and 51. As no abnormalities were detected, ophthalmic examinations were not conducted during the recovery phase.

ECGs were recorded before treatment began, during wk 13, 26, 39 and 51 of treatment, and wk 6 of the recovery phase, 1 and 24 h after dosing. Standard haematology and clinical chemistry parameters (Appendix 1) were analysed in blood samples collected pre-treatment, wk 13, 26, 39 and 52 of treatment, and wk 6 of the recovery phase. Water was withheld for approximately 5 h prior to the collection of urine before treatment began, during wk 12, 25, 38 and 51 of treatment, and wk 6 of the recovery phase, for the analysis of standard urinalysis parameters (Appendix 1). For toxicokinetic analysis, blood was sampled during wk 1, 13, 27 and 52 at 0, 0.5, 1, 3, 6, 12 and 24 h after the diet was offered. Dogs were killed after 52 week of treatment or following the 6-week recovery phase, then necropsied.

Standard organs were weighed (plus the lungs and salivary glands) and histopathologically examined (Appendix 2).

Results

MORTALITIES & CLINICAL SIGNS

One male (#417) from the 50000 ppm group was sacrificed in a moribund condition during the final week of the recovery period. This dog reportedly exhibited signs of "beagle pain syndrome" or idiopathic polyarteritis (e.g. painful neck, elevated temperature) and failed to respond to anti-inflammatory drugs. The findings for this dog and their time of onset were unrelated to treatment as the dog appeared normal throughout the 52-week treatment period. All other dogs survived to scheduled sacrifice.

Similar to other dog studies, pale coloured faeces occurred at 10000 and 50000 ppm. Loose stools were noted across all groups, including the control, but were not considered treatment-related.

BODY WEIGHT & FOOD CONSUMPTION

The mean pre-treatment bodyweight of the male control group was approximately 500 g higher than the three treatment groups and this difference was maintained through the study. However, mean bodyweight gain over 52-weeks was comparable between the control and treatment groups. There were a few dogs in the treated groups that showed a transient loss of bodyweight some time during the first 14 weeks of the study (male #395, 2000 ppm; male#397, 403 and 407, 10000 ppm; female #414, 50000 ppm). However, the authors' decision to increase the amount of feed from wk 15 based on the dogs appearing thin/lean is questionable because the majority of dogs actually gained weight over this period. There was no treatment-related effect on food consumption.

The mean achieved doses over 52 weeks of treatment were 83, 421 and 2058 mg/kg bw/day in males and 82, 406 and 2416 mg/kg bw/day in females at 5000, 15000 and 50000 ppm, respectively.

TOXICOKINETICS

Table 3.30 summarises the C_{max} and AUC_{24} values for Advantame and Advantame-acid. Mean C_{max} and AUC_{24} values for Advantame-acid increased over the dose range but generally showed a lack of proportionality, which became more evident as the study progressed. Females tended to have higher systemic exposure to Advantame/Advantame-acid. C_{max} and AUC_{24} were higher for Advantame-acid than Advantame indicating higher systemic exposure to the metabolite than the parent compound. Based on the average metabolite ratio, it is estimated that 99% of the administered dose of Advantame was converted to Advantame-acid prior to absorption.

TABLE 3.30: C_{MAX} AND AUC_{24} VALUES FOR ADVANTAME AND ADVANTAME-ACID IN DOGS.

Parameter	2000 ppm		10000 ppm		50000 ppm	
	Males	***Females***	***Males***	***Females***	***Males***	***Females***
Advantame						
C_{max} (ng/mL)						
d 1	4.68 (1)	5.85 (1)	30.0 (6.4)	35.3 96.0)	109 (23.3)	122 (20.9)
wk 13	4.13 (1)	7.67 (1)	19.6 (4.7)	14.3 (1.9)	69.4 (16.8)	151 (19.7)
wk 27	2.13 (1)	4.51 (1)	10.6 (5.0)	15.7 (3.5)	37.9 (17.8)	123 27.3)
wk 52	1.27 (1)	2.59 (1)	6.32 (5.0)	7.00 (2.7)	23.8 (18.7)	56.0 (21.6)
AUC_{24}						

Parameter	2000 ppm		10000 ppm		50000 ppm	
	Males	***Females***	***Males***	***Females***	***Males***	***Females***
(ng.h/mL)						
d 1	23.5 (1)	31.3 (1)	170 (7.2)	145 (4.6)	581 (24.7)	756 (24.2)
wk 13	24.2 (1)	28.3 (1)	73.4 (3.0)	61.2 (2.2)	353 (14.6)	755 (26.7)
wk 27	12.8 (1)	16.1 (1)	47.9 (3.7)	70.9 (4.4)	233 (18.2)	730 (45.3)
wk 52	6.93 (1)	7.01 (1)	37.8 (5.5)	37.3 (5.3)	148 (21.4)	388 (55.3)
Advantame-acid						
C_{max} (ng/mL)						
d 1	520 (1)	691 (1)	2610 (5.0)	2960 (4.3)	5560 (10.7)	6620 9 (9.6)
wk 13	699 (1)	682 (1)	2020 (2.9)	1580 (2.3)	3510 (5.0)	10300 (15.1)
wk 27	595 (1)	834 (1)	1260 (2.1)	1670 (2.0)	1990 (3.3)	3940 (4.7)
wk 52	350 (1)	544 (1)	1240 (3.5)	1200 (2.2)	2200 (6.3)	3270 (6.0)
AUC_{24} (ng.h/mL)						
d 1	5350 (1)	8010 (1)	28400 (5.3)	30300 (3.8)	42100 (7.9)	51400 (6.4)
wk 13	8750 (1)	10100 (1)	22300 (2.5)	22000 (2.2)	46900 (5.4)	130000 (12.9)
wk 27	6440 (1)	10000 (1)	15600 (2.4)	18600 (1.9)	27000 (4.2)	44200 (4.4)
wk 52	4470 (1)	8240 (1)	17900 (4.0)	13700 (1.7)	27500 (6.2)	39500 (4.8)
Metabolite ratio1						
d 1	245	280	166	246	76	75
wk 13	410	368	329	441	147	236
wk 27	517	736	365	316	119	70
wk 52	954	1324	645	415	196	133

Results expressed as the mean ± 1 sd; 1 = AUC_{24} for Advantame-acid ÷ AUC_{24} for Advantame

OPTHALMOSCOPY & ECG

There were no treatment-related ophthalmic abnormalities.

The mean heart rate of 50000 ppm males was elevated at all sampling intervals 1 h after dosing, but not significantly (see Table 3.31). The finding was attributable to a single dog (#417) that was tachycardic (heart rate >180 bpm) 1 h after dosing at all sampling points, and 24 h after dosing during wk 39. This was the same dog that was sacrificed in a moribund condition during the recovery phase due to an underlying inflammatory condition unrelated to treatment. Another 50000 ppm male (#419) was tachycardic 1 h after dosing during wk 39. Similar findings did not occur in females and there were no abnormalities in ECG intervals or waveforms. Further, an increase in heart rate was not evident in the 13-week dietary study using the same doses. On the basis of these considerations, the elevated heart rate in dog #417 is not considered treatment related.

TABLE 3.31: MEAN HEART RATE IN MALES 1H AFTER DOSING

Sampling Time	0 ppm	2000 ppm	10000 ppm	50000 ppm
Pretreatment	143±14	126±30	130±16	123±25
wk 13	113±20	123±21	104±15	130±26
wk 26	114±20	109±25	103±18	135±29
wk 39	110±19	117±28	99±14	146±43
wk 51	106±13	113±27	100±13	132±31

Results expressed as the mean beats/min ± 1 SD

HAEMATOLOGY

In the 13-week dog study by Powell and Scott (2005), significantly lower Hct, Hb and RBC were noted in both sexes at 15000 and 50000 ppm, which were considered unlikely to be treatment-related. For comparative purposes, these haematology parameters (in addition to reticulocytes) from the current study are summarised in Table 3.32. Variations in all parameters occurred over time, with similar patterns of change occurring in the treated and

control groups. In males, the significantly higher mean Hct and RBC during wk 13 at 50000 ppm are not attributable to treatment as these parameters were already significantly higher than the control prior to the commencement of dosing. In females during wk 52, mean Hct, Hb and reticulocytes were significantly higher ($p<0.05$) than the control at 10000 and 50000 ppm. As the mean values were consistent with those at other times, which were not statistically different to the control, these findings are not considered treatment- related.

TABLE 3.32: SELECTED HAEMATOLOGY FINDINGS

Parameter	Dietary concentration (ppm)							
	0		1500		10000		50000	
	♂	♀	♂	♀	♂	♀	♂	♀
Hct (L/L)								
Pretreatment	0.413	0.431	0.419	0.450	0.427	0.452	0.441*	0.454
Wk 13	0.407	0.446	0.426	0.451	0.412	0.455	0.440*	0.475
Wk 26	0.426	0.420	0.432	0.452	0.425	0.448	0.431	0.449
Wk 39	0.427	0.437	0.436	0.472	0.435	0.445	0.445	0.452
Wk 52	0.422	0.421	0.450	0.455	0.429	0.465*	0.436	0.455*
Recovery	0.420	0.393	-	-	0.447	0.435	0.451	0.461
Hb (g/dL)								
Pretreatment	14.2	14.8	14.2	15.3	14.4	15.3	14.8	15.2
Wk 13	13.7	14.8	14.2	15.1	13.6	15.0	14.7	15.7
Wk 26	14.6	14.3	14.7	15.5	14.3	15.1	14.6	15.4
Wk 39	14.4	14.8	14.5	16.0	14.5	14.9	14.9	15.1
Wk 52	14.8	14.8	15.5	16.0	15.0	16.1*	15.2	15.9*
Recovery	14.0	13.6	-	-	15.1	14.5	15.3	15.7
RBC ($x10^{12}$/L)								
Pretreatment	6.07	6.41	6.23	6.62	6.35	6.58	6.49*	6.59
Wk 13	6.01	6.62	6.42	6.63	6.28	6.70	6.60**	6.99
Wk 26	6.38	6.33	6.61	6.73	6.57	6.72	6.57	6.65
Wk 39	6.31	6.48	6.58	6.88	6.59	6.52	6.62	6.52
Wk 52	6.32	6.38	6.96	6.74	6.73	7.02	6.66	6.75
Recovery	5.84	5.64	-	-	6.66	6.20	6.34	6.51
Reticulocytes (%)								
Pretreatment	0.55	0.44	0.59	0.67	0.58	0.52	0.59	0.45
Wk 13	0.67	0.64	0.82	0.80	0.79	0.60	0.67	0.63
Wk 26	1.09	0.50	1.32	0.77	1.25	0.52	0.85	0.60
Wk 39	0.79	0.47	1.00	0.71	0.90	0.46	0.76	0.50
Wk 52	0.65	0.26	1.01	0.59	0.86	0.49*	0.71	0.73**
Recovery	0.34	0.27	-	-	1.04	0.35	0.29	0.31

Results expressed as the mean; *p<0.05; **p<0.01

CLINICAL CHEMISTRY & URINALYSIS

There were no treatment-related clinical chemistry or urinalysis findings.

ORGAN WEIGHTS, MACROPATHOLOGY & HISTOPATHOLOGY

Selected mean absolute organ weights for females sacrificed after 52 weeks of treatment are presented in Table 3.33. The mean absolute right ovary weight across all treated groups was significantly lower than the control. The mean relative right ovary weight was also lower than the control at 2000 and 50000 ppm. In the absence of a dose-response relationship or any abnormal histopathology, these differences are not considered treatment related. Mean absolute and relative spleen weights were lower than the control in all treated groups but in the absence of a similar difference in males or any corroborative evidence of an effect on this organ (e.g. histopathology or haematology) these differences are not considered treatment-related. The mean weight of the uterus and cervix was noticeably lower in treated

groups relative to the control, particularly at the highest dose where the uterus plus cervix were at least half that of the control group. However, given the large variability of the data and the absence of any histopathology, these differences are not considered treatment-related.

TABLE 3.33: ORGAN WEIGHTS IN FEMALE DOGS (WEEK 52)

Sampling Time	0 ppm	2000 ppm	10000 ppm	50000 ppm
Terminal bw (g)	11225	11950	11125	10200
Right ovary (g)	0.99±040 (0%)	0.83±0.42* **(-16%)**	0.52±0.10** **(-47%)**	0.66±0.25** **(-43%)**
Right ovary (%)	0.00881 (0%)	0.00694 **(-21%)**	0.00898 **(+2%)**	0.00647 **(-27%)**
Spleen (g)	119.9±41.1 (0%)	85.8±20.9 **(-28%)**	90.2±25.4 **(-25%)**	76.2±30.3 **(-36%)**
Spleen (%)	1.068 (0%)	0.718 **(-33%)**	0.811 **(-34%)**	0.567 **(-47%)**
Uterus + cervix (g)	18.6±9.2 (0%)	14.0±8.6 **(-25%)**	9.9±11.9 **(-47%)**	7.3±5.6 **(-61%)**
Uterus + cervix (%)	0.166 (0%)	0.117 **(-30%)**	0.0890 **(-46%)**	0.0716 **(-57%)**

Absolute organ weights are expressed as the mean (g) ± 1 SD, with the % difference (+ or -) to the control given in parentheses (bolded); *p≤0.05; **p≤0.01; relative organ weights are expressed as % of terminal bodyweight and were calculated by dividing the mean absolute organ weight by the mean terminal bodyweight then multiplying by 100.

There was no treatment-related effect on the incidence or severity of macroscopic or histopathogical abnormalities.

Conclusions

The NOAEL was 50000 ppm (equal to 2058 mg/kg bw/day in males and 2416 mg/kg bw/day in females) based on the lack of any adverse effect at this dietary concentration.

3.2.7 Genotoxicity Studies

May K, Watson D & Burton DA (2001) ANS9801. Bacterial mutation assay. Report No. AJO 154/012404. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. **GLP**: UK, OECD, EC, Japan, US FDA. **QA statement**: Yes. **Guidelines**: OECD (Test Guideline 471), EEC, US EPA, US FDA, Japan, JMHW & Official Notice of J MOL.

Clare MG, Kernahan J & Clemson AD (2002) ANS9801. Mammalian cell mutation assay. Report No. AJO 159/013035. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. **GLP**: UK, OECD, EC, Japan, US FDA. **QA statement**: Yes. **Guidelines**: OECD (Test Guideline 476), US EPA & EEC.

Mehmood Z, Pritchard L, Clemson AD & Davison C (2001) ANS9801. Mouse micronucleus test. Report No. AJO 160/013188. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. **GLP**: UK, OECD, EC, Japan & US FDA. **QA statement**: Yes. **Guidelines**: OECD (Test Guideline 474), EC, US EPA & US FDA.

Two *in vitro* studies and one *in vivo* genotoxicity study were submitted as part of the current Application. These studies were GLP compliant and conducted according to appropriate test guidelines. Signed QA statements were contained in the respective study reports. The two *in vitro* studies were conducted in the presence and absence of an exogenous source of metabolic activation (S9 liver preparations from Aroclor 1254-induced rats). Positive and negative (vehicle) controls were tested in each study and gave expected results.

Advantame showed no evidence of mutagenic or clastogenic activity in these assays (Table 3.34).

TABLE 3.34: SUMMARY OF GENOTOXICITY STUDIES

Test	Test system	Test article	Concentration or dose range	Result	Reference
Bacterial reverse mutation (Ames test)	*S. typhimurium* strains TA98, TA100, TA1535 & TA1537. *E. coli* WP2 uvrA pKM 101 (±S9)	Advantame (99.2% purity; Lot No. 000530) DMSO vehicle	5- 5000 µg/plate	Negative No cytotoxicity	May *et al* (2001)
Mammalian forward mutation	Mouse lymphoma L5178Y cells 3 h (±S9) & 24 h (-S9) treatment	Advantame (100.1% purity; Lot No. 000825) DMSO vehicle	Preliminary toxicity test: 39-5000 µg/well Main test: 500-5000 µg/well	Negative Cytotoxicity[1]	Clare *et al* (2002)
Mouse micronucleus	Mice (CD-1 strain) PO, gavage 24 and 48 h sampling times	Advantame (100.1% purity; Lot No. 000825) 1% methyl cellulose vehicle	Preliminary toxicity test: 2000 mg/kg bw (2 mice/sex) 500, 1000 & 2000 mg/kg bw (7 males/group)	Negative Toxicity[2]	Mehmood *et al* (2001)

1 = Preliminary toxicity test: cytotoxicity at and above 2500 µg/well -S9 and at and above 625 µg/well +S9 (3h treatment), and at and above 625 µg/well (24 h treatment); Main study: cytotoxicity at and above 1250 µg/well (3 h treatment) and 500 µg/well (24 h treatment).

2 = In the preliminary toxicity test, all mice exhibited hunched posture 90 min after dosing; no clinical signs were observed in the main test. At the 48 h sampling time, the mean proportion of immature erythrocytes in the high-dose group was significantly lower ($p<0.01$) than the control (41 *versus* 46%, respectively; range 38-49% and 44-49%). However, given the relatively small magnitude of this difference and that no difference was evident at 24 h, it is not considered toxicologically significant.

3.2.8 Reproduction & Developmental Toxicity Studies

3.2.8.1 Rats

Willoughby CR (2002) ANS9801. Study of effects on embryo-fetal development in CD rats treated by dietary administration. Report No. AJO 182/014156. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. **GLP**: UK, OECD, EC, Japan, US FDA. **QA statement**: Yes.

Experimental

Advantame (99.9% purity; Batch No. 000825; sourced from the Sponsor) was admixed in the diet and fed *ad libitum* to 22 pregnant Crl:CD (SD) IGS BR rats [10-11 weeks old; 231-285 g bw; originally sourced from Charles River (UK) Ltd (Margate, Kent) & mated 1:1 with males of the same strain] at concentrations of 0, 5000, 15000 or 50000 ppm from d 0-20 of gestation. The dose selection was based on the results of a pilot developmental toxicity study (Report No. AJO165/010069).

Dams were observed at least twice daily for clinical signs, with a more thorough physical examination conducted on d 0, 5, 12, and 18 of gestation. Maternal bodyweight was recorded on d 0, 3, 6, 10, 14, 18 and 20. Food consumption was recorded on d 0-2, 3-5, 6-9, 10-13, 14-17 and 18-19. Dams were killed on d 20 of gestation and macroscopically examined. The gravid uterus, ovaries and pituitary were weighed. The following parameters were recorded: number of corpora lutea, implantation sites, resorption sites and the distribution of foetuses in each uterine horn. Each foetus was weighed, sexed and macroscopically examined. Approximately half of the foetuses were examined for skeletal abnormalities, with the remainder examined for visceral abnormalities.

Results

The achieved doses of Advantame over the duration of the study are summarised in Table 3.35.

TABLE 3.35: ACHIEVED DOSES OF ADVANTAME (mg/kg bw/day)

Gestation days	5000 ppm	15000 ppm	50000 ppm
0-2	500	1498	4370
3-5	503	1495	5215
6-9	481	1472	5219
10-13	456	1433	5039
14-17	443	1376	4675
18-19	382	1132	4036
Mean	465	1418	4828

All dams survived to scheduled sacrifice. Discolouration of the faeces (pale, dark, striped, green or purple blue) was the only treatment-related sign occurring across all treated groups at varying incidences. At 50000 ppm, pale faeces predominated (all rats affected by d 20), while at 5000 and 15000 ppm green faeces was the main finding (up to 8 rats affected/group). These signs are considered attributable to the high concentration of Advantame and/or its metabolites in the digestive tract and are not evidence of toxicity.

There was no treatment-related effect on mean absolute bodyweight. Mean cumulative body weight gain was significantly lower ($p<0.01$ or 0.05) than the control at 50000 ppm (30, 23, 16, 13, 11 and 9% lower at d 0-3, 0-6, 0-10, 0-14, 0-18 and 0-20, respectively). When adjusted for gravid uterine weight, mean bodyweight gain from gestation d 0-20 was significantly lower (~16%; $p<0.01$) than the control at 50000 ppm. There was no difference in bodyweight gain at 5000 and 15000 ppm compared to the control. In 50000 ppm dams, mean food consumption was initially significantly lower (~12%; $p<0.01$) than the control (d 0-2) but thereafter was significantly higher (~7%; $p<0.01$ or 0.05). At 15000 ppm, mean food consumption was significantly higher than the control (~7%) during d 10-13 and 14-17. The authors suggested that the initially low food consumption at 50000 ppm was due to the poor palatability of the diet. Thereafter, rats adapted to the taste and increased their food consumption but never regained their initial bodyweight deficit relative to the control group. It is noted that the authors considered these findings adverse and used them as the basis of the NOAEL for the study even though they described the effects as “slight”. The reviewing FSANZ toxicologist considers that while these findings are clearly treatment-related, they are unlikely to represent a direct toxicological effect of the test material but reflect the high proportion of Advantame in the diet and its consequent effect on palatability and food consumption. On this basis, these treatment-related effects on food consumption and bodyweight are not considered toxicologically significant.

There was no treatment-related effect on the weight of the gravid uterus, ovaries or pituitary. Litter parameters and placental, litter or fetal weights were unremarkable. No treatment-related visceral or skeletal abnormalities were detected.

Conclusion

Advantame was not teratogenic. The NOAEL for maternal, fetal and developmental toxicity was 50000 ppm (equal to 4828 mg/kg bw/day in dams), the highest dose tested.

Willoughby CR (2004) ANS9801. Study of reproductive performance in CD rats treated continuously through two successive generations by dietary administration. Report No. AJO 203/033888. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. **GLP**: UK, OECD, EC, Japan, US FDA. **QA statement**: Yes.

Experimental

Advantame (98.6% purity; Batch No. 010713; sourced from the Sponsor) was admixed in the diet and fed *ad libitum* to Crl:CD (SD) IGS BR rats [sourced from Charles River (UK) Ltd, Margate, Kent] at concentrations of 0, 2000, 10000 or 50000 ppm throughout all phases of pre-mating (~10 weeks), mating, gestation and lactation for two successive generations. The F0 and F1 parental generations consisted of 30 and 25 rats/sex/group, respectively. Litter sizes were adjusted to 10 offspring 4 days after birth.

Observations for mortalities and clinical signs were made daily, with a more detailed clinical examination performed weekly. Bodyweight and food consumption were recorded weekly during the pre-mating period and in females at 0, 6, 13 and 20 days after mating, and at d 1, 4, 7, 14 and 21 of lactation. The oestrus cycle was determined for each female 22 days prior to mating and at 28 days post-partum by examining vaginal smears. All rats were weighed prior to necropsy. Males and females were mated 1:1 for up to 3 weeks. From d 20 after mating, dams were inspected thrice daily for evidence of parturition and the numbers of live and dead offspring recorded. The following litter parameters were recorded: clinical signs; litter size (d 1-21 and d 25); sex ratio (d 1, 4 and 21); and bodyweight (d 1, 4, 7, 14, 21, 21 and 25). Prior to weaning, the following reflex developmental tests were performed on offspring: surface righting, air righting, auditory function and visual function. Twenty F1 offspring/sex/group were subjected to the following sensory examinations: motor activity; neuromuscular function; learning ability.

F0 and F1 parental males were killed at weaning (after 16-17 weeks of treatment), while dams were killed on d 28 post-partum. Dams failing to mate or produce a viable litter were killed 25 days after mating. Dams whose litter died before d 21 were killed on the day on which the last offspring died. All F0 and F1 adults were necropsied. The number of implantation sites was recorded for each dam, while the appearance of mammary tissue was examined in females that had lost a litter. Standard reproduction indices were calculated (Appendix 3). F1 and F2 offspring were macroscopically examined including those culled on post-partum d 4, those dying prematurely and those unselected offspring killed at 30 days of age. Following sacrifice, sperm from control and 50000 ppm F0 and F1 males was examined for the following: motility; morphology; sperm count and spermatid count.

With the exception of the thyroid gland, standard organ weights were recorded (Appendix 2) for all surviving F0 and F2 parental rats. Brain, spleen and thymus weights were recorded from two F1 and F2 rats/sex/litter. The following organs/tissues from ten F0/F1 adults in the control and 50000 ppm groups were histopathologically examined: adrenals, brain, epididymis, kidneys, liver, ovaries, seminal vesicles, uterus, vagina and any macroscopically abnormal tissue. The reproductive organs of rats showing signs of reduced fertility (e.g. failing to mate) were also histopathologically examined.

Results

PARENTAL RATS

Achieved doses of Advantame are summarised in Table 3.36, with females exposed to marginally more Advantame than males during the pre-mating period. Achieved doses in dams during lactation were approximately double that during pre-mating and gestation.

TABLE 3.36: ACHIEVED DOSES OF ADVANTAME (mg/kg bw/day)

Time period	2000 ppm		10000 ppm		50000 ppm	
	Males	Females	Males	Females	Males	Females
F0 generation						
Premating	164	184	833	907	4410	4776
Gestation	-	163	-	795	-	4136
Lactation	-	320	-	1575	-	8192
F1 generation						
Premating	204	229	1036	1139	5431	5920
Gestation	-	167	-	865	-	4457
Lactation	-	316	-	1592	-	8447

Four treated F0 females were killed *in extremis* 21-24 days after mating due to parturition difficulties (0/30, 1/30, 2/30 and 1/30 at 0, 2000, 10000 and 50000 ppm, respectively). A single F1 female from the 2000 ppm group was killed *in extremis* after weaning. In the absence of a dose-response relationship and given the inconsistency between the F0 and F1 generation, these deaths are not considered to be associated with treatment. Treatment-related signs were confined to green staining of the faeces at 2000 and 10000 ppm in F0 parents (up to 3 and 21 rats/sex/group, respectively), and at above 2000 ppm in F1 parents (up to 5, 20 and 7 rats/sex/group, respectively). Purple staining of the faeces was also reported in a smaller number (<8) of F0 and F1 parents at 10000 and 50000 ppm. White faeces were reported in the majority of F0 and F1 parents at 50000 ppm. Similar observations were recorded for dams during gestation. These observations are not considered to represent a toxicological effect.

There was no treatment-related effect on absolute bodyweight, bodyweight gain or food conversion efficiency in F0/F1 parental rats during the pre-mating period, and in dams during gestation and lactation. Food consumption was unaffected by treatment in F0 parents but was significantly higher than the control ($p<0.01$ or 0.05) at 50000 ppm in F1 parents at most weeks during the pre-mating period (6-9% higher in males and 7-12% higher in females). In the absence of a consistent effect between F0 and F1 parents or any effect on food conversion efficiency, these statistical differences are not considered treatment-related. There was no treatment-related effect on food consumption in dams during gestation and lactation.

There was no difference in the oestrus cycle between treated and control F0/F1 females before mating and prior to termination. Sperm analysis of F0/F1 males was unremarkable. In F0 males, mean absolute and relative prostate weights were somewhat lower than the control at 10000 and 50000 ppm (~6 and 10%, respectively), but not significantly. In F1 males, mean absolute and relative prostate weights were also lower (~20%) than the control in the 50000 ppm group, but not significantly. However, at 2000 and 10000 ppm, absolute prostate weight was approximately 10% higher than the control group, while relative prostate weight was approximately 7% higher than the control at 10000 ppm. Given the lack of a dose-response relationship, absence of statistical differences and inconsistent results, these differences in prostate weight are not considered treatment-related.

In F0 females, the mean absolute and relative weights of the uterus and oviducts were

significantly lower (~20%; p<0.01) than the control at 50000 ppm. In the absence of a similar finding in F2 females, any pathology of this organ or effect on fertility, this finding is not considered treatment-related. The mean absolute and relative weights of the ovaries were approximately 30% lower than the control across all treatment groups, but not significantly (absolute weights: 0.178±0.282, 0.127±0.018, 0.125±0.021 and 0.123±0.018 g at 0, 2000, 10000 and 50000 ppm, respectively). However, these differences are attributable to a single outlying control rat with an unusually large right ovary (0.3589 g).

There were no treatment-related macroscopic or histopathological abnormalities detected in F0/F1 rats.

REPRODUCTION PARAMETERS

There was no treatment-related effect on mating performance or fertility, gestation length, litter size, survival indices or sex ratio.

OFFSPRING

At 50000 ppm, the mean bodyweights of F1 offspring were significantly higher (p<0.05) than the control group four days after birth (~9% in males and 8% in females) but were similar over the remainder of the lactation period. At sexual maturation the mean bodyweight of F1 males in the 50000 ppm group was approximately 7% lower than the control (p<0.05). Based on the inconsistency of these results and that no significant bodyweight differences between treated and control groups occurred in F2 offspring, these findings are not considered treatment-related.

Pre-weaning reflex developmental tests were unremarkable in F1/F2 offspring.

There was no treatment-related effect on mean or absolute organ weights in F1/F2 offspring.

While two of the 10 sampling intervals in the motor activity assessment detected significantly lower or higher motor activity in F1 males, the inconsistency of the results and that total motor activity was unremarkable indicates the absence of any association with treatment. In a similar manner, the significantly lower (p<0.05) motor activity in F1 females at 10000 and 50000 ppm at a single sampling interval is not considered to represent a treatment-related effect. Rotarod performance of F1 offspring was unaffected by treatment. In the Morris maze test, the trial time of F1 females during d 1 at 10000 and 50000 ppm was significantly lower (p<0.05) than the control (63.7, 57.8, 49.3 and 58.2 seconds at 0, 2000, 10000 and 50000 ppm, respectively) but in the absence of a dose-response relationship, any differences in performance on d 2 or 3, or a similar finding in males, these significant differences are not considered to indicate a treatment-related effect.

Sexual maturation in F1 females was normal, while there was delay of 1 or 2 days in sexual maturity (i.e. balano-preputial separation) in F1 males, which was statistically significant (p<0.05) at every dietary concentration. However, given that the mean values for all treated groups fell with the historical control range of 40-76 days for balano-preputial separation for male CD rats (CRL 2002), such a minor delay in this study is not considered biologically relevant.

Conclusions

The NOAEL for parental, offspring and reproductive toxicity was 50000 ppm (equivalent to 5431/5920 mg/kg bw/day in F1 males/females), the highest dose tested.

3.2.8.2 Rabbits

Fulcher SM, Renaut SD & Bottomly AM (2002) ANS9801: Preliminary embryo-fetal toxicity in the rabbit by gavage administration. Second Amended Final. Report No. AJO 183/010158. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. **GLP**: UK. **QA statement**: No. **Guidelines**: US FDA. [Report amended in 2002 and 2005]

* Baldrey SF, Phelan MR, Holding J, Carr MJ & Cook SC (2002g) Toxicokinetic Report. ANS9801: Preliminary embryo-fetal toxicity in the rabbit by gavage administration. Report No. AJO 183/010158.

Experimental

Advantame (>99% purity; Batch No. 000825; sourced from the Sponsor) in 1% (w/v) methylcellulose was administered by gavage to groups of 6 pregnant New Zealand White (NZW) rabbits at 0, 500, 1000 or 2000 mg/kg bw/day from d 6-28 of gestation. The dose volume was 5 mL/kg bw. Rabbits were sourced from Highgate Farm (Lincolnshire, England), approximately 18-22 weeks old and acclimatised for 26 days prior to 1:1 mating with males of proven fertility. The bodyweight range was 3.59-4.62 kg at allocation to the treatment groups. Rabbits were housed individually under standard conditions, with food and water available *ad libitum*.

Dams were observed daily for clinical signs. During the dosing period, more detailed observations were made pre-dose, at the end of dosing and at 0.5, 1, 2 and 4 h after dosing or as late possible during the day. Bodyweight and food consumption were recorded daily. Water consumption was recorded on d 1, 8, 13, 20 and 26. For toxicokinetic analysis, blood was sampled on d 6 (the first day of dosing) and 27; Advantame and Advantame-acid were analysed by LC/MS/MS. Surviving dams were sacrificed on d 29 and necropsied. The following maternal/litter parameters were recorded gravid uterine weight; number of corpora lutea; number of implantation sites; number of early and late resorptions; number and distribution of live and dead foetuses in each uterine horn. The following fetal parameters were recorded: weight; placenta weight; external abnormalities sex. Half of the foetuses were processed for the examination of skeletal abnormalities and the other half for visceral abnormalities.

Results

TOXICOKINETICS

Mean C_{max} and AUC_{24} values for Advantame and Advantame-acid at d 6 and 27 are presented in Table 3.37 but are viewed as estimates only due to the underestimation of the Advantame-acid concentration. Systemic exposure to Advantame-acid was higher than to Advantame as shown by the larger C_{max} and AUC_{24} values, with the large metabolite ratios indicating extensive metabolism to Advantame-acid. Based on the average metabolite ratio, it is estimated that a minimum of 97% of the administered dose of Advantame was converted to Advantame-acid prior to absorption. Given the underestimation of Advantame-acid due to the analytical uncertainty, the actual level of GIT conversion was probably closer to 100%.

TABLE 3.37: TOXICOKINETIC PARAMETERS IN RABBITS

Parameter	500 mg/kg bw/day	1000 mg/kg bw/day	2000 mg/kg bw/day
Advantame			
C_{max} (ng/mL)			
d 6	944 (1)	714 (0.8)	1070 (1.1)
d 27	847 (1)	1178 (1.4)	2980 (3.5)
AUC_{24} (ng.h/mL)			
d 6	4982 (1)	3737 (0.8)	8382 (1.7)
d 27	3906 (1)	11594 (3.0)	22367 (5.7)
Advantame-acid			
C_{max} (ng/mL)			
d 6	10513 (1)	13694 (1.3)	18923 (1.8)
d 27	14338 (1)	21382 (1.5)	30400 (2.1)
AUC_{24} (ng.h/mL)			
d 6	151896 (1)	117112 (0.8)	274417 (1.8)
d 27	147414 (1)	268999 (1.8)	486206 (3.3)
Metabolite ratio[2]			
d 6	31	31	33
d 27	38	23	22

Results are expressed as the mean, with the C_{max} and AUC_{24} ratios contained in parentheses; 1 = AUC_{24} d 27 ÷ AUC_{24} d 6; 2 = AUC_{24} Advantame-acid ÷ AUC_{24} Advantame

MORTALITIES & CLINICAL SIGNS

At 2000 mg/kg bw/day, one dam (#24) showed marked reddening and swelling of the ano-genital region and was killed on d 17. Necropsy revealed that this dam was pregnant and had a pronounced and reddened labia. One control dam and one dam from the 500 mg/kg bw/day group (#10) were killed on d 20 as both showed evidence of abortion.

Green and/or purple and/or pink staining of the cage lining was observed for the majority of treated dams (3/6, 5/6 and 6/6 dams at 500, 1000 and 2000 mg/kg bw/day, respectively). Green and/or purple urine was observed directly in three mid-dose dams, while one high-dose dam (#24) had green bladder contents. Green staining of the paws and tail were observed in the majority of dams at 1000 and 2000 mg/kg bw/day, with green staining of the perigenital region also occurring in some animals. In the single high-dose dam (#24) that was killed, green staining of the whole body was also observed. The compound or compounds responsible for this green/purple colouration were not identified. Pale faeces were observed in 2 and 4/6 dams at 1000 and 20000 mg/kg bw/day; no other colouration of the faeces was recorded.

BODYWEIGHT & FOOD/WATER CONSUMPTION

Group mean bodyweight and bodyweight gain (both adjusted for gravid uterus weight), food consumption and water consumption were unaffected by treatment. An examination of individual bodyweight and food consumption data revealed a marginal loss of bodyweight several days prior to sacrifice of the single high-dose dam (#24). This same dam also had reduced food and water consumption from d 12 or 13.

NECROPSY

Gravid uterine weights were unremarkable. The only treatment-related macroscopic finding was the presence of dark or green bladder contents in a few treated dams (two dams each at 500 and 2000 mg/kg bw/day). The caecum of 1, 1 and 3 dams at 500, 1000 and 2000 mg/kg bw/day contained gaseous and dark contents. None of these findings are considered adverse.

LITTER PARAMETERS

Litter parameters are summarised in Table 3.38 and indicate that there was no treatment-related effect on any parameter.

TABLE 3.38: SUMMARY OF REPRODUCTIVE PERFORMANCE OF DAMS

Parameter	Dose (mg/kg bw/day)			
	0	500	1000	2000
Mated	6	6	6	6
Pregnant	5	6	6	5
Aborted	1	1	0	0
Killed (pregnant)	0	0	0	1
Killed (not pregnant)	0	0	0	0
Surviving dams with live young (d 29)	4	5	6	4
Litter size (live young)[1]	8.3±1.7	5.8±3.5	11.0±2.1	8.3±3.6
Late resorptions[2]	2.5	1.6	0.7	2.5
Post-implantation loss (%)	19.6	17.6	5.3	25.8
Gravid uterine weight (kg)[1]	0.52±0.1	0.41±0.2	0.64±0.1	0.51±0.2

1 = mean ± 1 SD; 2 = expressed as the mean from the dams with live young at d 29

FETAL PARAMETERS

There was no treatment-related effect on any fetal parameters.

Conclusion

The NOAEL for maternal and developmental toxicity was 2000 mg/kg bw/day, the highest dose tested.

Fulcher SM, Renaut SD & Bottomly AM (2003) ANS9801: Study of effect on embryo-fetal toxicity in the rabbit by gavage administration. Report No. AJO 190/022479. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. **GLP**: UK, EC, OECD, US FDA & Japan. **QA statement**: Yes. **Guidelines**: US FDA.

Experimental

Advantame (>99% purity; Batch No. 001212; sourced from the Sponsor) in 1% (w/v) methylcellulose was administered by gavage to groups of 24 pregnant New Zealand White (NZW) rabbits at 0, 500, 1000 or 2000 mg/kg bw/day from d 6-28 of gestation. The dose volume was 5 mL/kg bw. The dose selection was based on the results of a preliminary developmental toxicity study (Report No. AJO183/010158), where doses up to 2000 mg/kg bw/day were well-tolerated. Rabbits were sourced from Highgate Farm (Lincolnshire, England), approximately 17-23 weeks old and acclimatised for 12 days prior to 1:1 mating with males of proven fertility. The bodyweight range was 3.15-4.70 kg at allocation to the treatment groups. Rabbits were housed individually under standard conditions, with food and water available *ad libitum*.

Dams were observed daily for clinical signs. During the dosing period, more detailed observations were made pre-dose and at 0.5, 1, 2 and 4 h after dosing or as late possible

during the day. Bodyweight and food consumption were recorded daily. Surviving dams were sacrificed on d 29 and necropsied. The following litter parameters were recorded gravid uterine weight; number of corpora lutea; number of implantation sites; number of early and late resorptions; number and distribution of live and dead foetuses in each uterine horn. The following fetal parameters were recorded: weight; placenta weight; external abnormalities sex. Half of the foetuses were processed for the examination of skeletal abnormalities and the other half for visceral abnormalities.

Results

MORTALITIES & CLINICAL SIGNS

Seven dams were killed *in extremis* prior to scheduled sacrifice (1, 0, 1 and 5 at 0, 500, 1000 and 2000 mg/kg bw/day, respectively). At 2000 mg/kg bw/day, one dam (#93) showed hunched and unsteady posture, hypoactivity, low muscle tone, weight loss (570 g from d 9) and reduced food consumption, and was killed on d 17. Four other high-dose dams (#75, 82, 92 and 96) were killed at later times (d 23-26). These rabbits lost bodyweight several days before sacrifice (350, 490, 500 and 200 g, respectively) and had reduced faecal output and/or loose stools. However, reduced faecal output and/or loose stools were observed across all groups of survivors (including the control) and therefore this finding is not attributable to treatment. Low food and water consumption were also noted in some of these dams. At 1000 mg/kg bw/day, one non pregnant dam (#52) exhibited collapsed posture, loss of co-ordination and locomotion, vocalisation and respiratory difficulties, and was sacrificed on d 27. Bodyweight loss (710 g) was recorded from d 25 to sacrifice. A single control dam (#1) was killed on d 17 shortly after dosing due to “convulsive-like signs, vocalisation and respiratory distress”. No other abnormalities were observed in this dam.

Green, purple or pink staining of the cage tray paper was observed for the majority of treated dams, with green/purple/blue or pink urine observed in 7, 13 and 9/24 at 500, 1000 and 2000 mg/kg bw/day, respectively. No green or purple colouration of the faeces was reported for any dam.

Green staining of external surfaces (muzzle, ventral body surface, urinogenital, region, all paws and tail) in all mid- and high-dose decedants, and in 1, 6 and 7 surviving dams at 500, 1000 and 2000 mg/kg bw/day, respectively, were observed.

BODYWEIGHT & FOOD CONSUMPTION

There was no significant difference in mean absolute bodyweight between treated and control groups. At 2000 mg/kg bw/day, mean bodyweight gain was lower than the control from d 6-8, 6-10 and 6-12 (0, 14 and ~50% of the control, respectively), with the latter significantly different ($p \leq 0.05$) to the control. From d 12, bodyweight gain recovered to be comparable to the control group; there was no difference in mean bodyweight gain (both with and without correction for gravid uterine weight) from d 6-29 to the control group. There was no treatment-related effect on absolute bodyweight or bodyweight gain at the two lower doses. When adjusted for gravid uterine weight, there was no difference in mean absolute bodyweight at d 29 or bodyweight gain from d 6-29.

There was no treatment-related effect on food consumption in survivors. While water consumption was not quantified, low water consumption was observed in the majority of decedants (including the single control dam). In survivors, low water consumption was observed across all groups (including the control) and is therefore not considered treatment-related (5/19, 8/18, 4/21 and 5/13 dams at 0, 500, 1000 and 2000 mg/kg bw/day, respectively).

NECROPSY

Green bladder contents were observed in 1, 2 and 3 dams at 500, 1000 and 2000 mg/kg bw/day, respectively, which includes one of the high-dose dams killed prior to the end of the treatment period (#82). In one high-dose survivor, an enlarged bladder with green/brown contents was observed. These findings are considered treatment-related and may be due to a coloured metabolite of Advantame-acid in urine.

In high-dose dams sacrificed on d 17 or 23-26, green or dark GIT contents were observed in the stomach (#92), caecum (#93 & 96), colon and rectum (#82 & 96). In some dams, the caecum was distended (#82) or its wall was haemhorragic (#75). In some instances the GIT contents were described as viscous (#82 & 92) or gelatinous and frothy (#75). Individual high-dose dams had gall bladder enlargement and a green-tinged serosa of stomach (#92), or punctate cysts on both kidneys (#75).

In the mid-dose dam that was sacrificed in a moribund condition (#52), the internal surfaces of the kidneys were stained green, with punctate foci also observed on both kidneys. Consistent with the high-dose group, green GIT contents were observed. A thick, green gelatinous material was also present at the ileo-caecal junction. Additionally, thickening of the stomach wall and a slightly haemorrhagic caecal wall were noted. The similarity of these observations to those at the high-dose indicates a probable relationship to treatment.
A pale liver was noted in two high-dose dams, including one sacrificed in extremis (#96) and one that aborted its litter on d 29 (#84). The latter dam also had dark and fluid caecal contents. Three mid-dose survivors also had pale livers.

LITTER PARAMETERS

The performance of the dams is summarised in Table 3.39. One control (#10) and one high-dose dam (#84) aborted their litters on d 21 and 29, respectively. The control dam lost 170 g bodyweight from d 20 and necropsy revealed no abnormalities. The high-dose dam lost 270 g bodyweight from d 26. Necropsy revealed that the majority (3/4) of its remaining foetuses were dead, placentae were pale and mottled, and there was a “large amount of serous red fluid in the uterus and cervix”. Other abnormalities included pale liver, dark caecal contents and absent faecal pellet formation.

Mean litter size was lower than the control at 1000 mg/kg bw/day but only marginally so at 2000 mg/kg bw/day. The lack of a dose-response relationship or any effect on litter weight indicates that this finding was not treatment-related. The number of late resorptions was ~3-fold higher than the control at 2000 mg/kg bw/day. No whole-litter resorptions were observed. There was a dose-related increase in post-implantation loss, which was approximately 2-fold higher than the control at and above 1000 mg/kg bw/day. However, given that there was no treatment-related effect on litter size, the relative increases in late resorptions and post-implantation loss are considered incidental findings.

TABLE 3.39: SUMMARY OF REPRODUCTIVE PERFORMANCE OF DAMS

Parameter	Dose (mg/kg bw/day)			
	0	500	1000	2000
Mated	24	24	24	24
Pregnant	21	18	22	18
Aborted	1	0	0	1
Killed (pregnant)	1	0	0	4
Killed (not pregnant)	0	0	1	1

Parameter	Dose (mg/kg bw/day)			
	0	500	1000	2000
Surviving dams with live young (d 29)	19	18	21	13
Litter size (live young)[1]	8.2±2.5	8.6±2.1	6.0±2.8	7.7±2.7
Late resorptions[2]	0.3	0.4	0.4	1.0
Post-implantation loss (%)	5.6	8.0	10.5	12.3
Gravid uterine weight (kg)[1]	0.51±0.2	0.54±0.1	0.43±0.1	0.51±0.1

1 = mean ± 1 SD; 2 = expressed as the mean from the dams with live young at d 29;

FETAL PARAMETERS

There was no treatment-related effect on any parameters, including the incidence of skeletal or visceral abnormalities.

Conclusions

The NOAEL for maternal toxicity was 500 mg/kg bw/day, based on the occurrence of clinical signs and mortalities at and above 1000 mg/kg bw/day. The NOAEL for developmental toxicity was 2000 mg/kg bw/day, the highest dose tested.

3.2.9 Neurotoxicity Studies

No specific neurotoxicity studies were submitted. However, no evidence of neurotoxicity was evident in numerous studies across multiple species including studies incorporating standard neurotoxicity endpoints into their design (13-week rat study by Chase et al 2004b; rat reproduction study by Willoughby 2004; acute rat study by Williams & Adams 2001).

3.2.10 Human Studies

*Warrington S (2004) An open, dose escalating study to determine the safety, tolerability and pharmacokinetic profile of ANS9801 in health male volunteers. Study No. ANSE-101. Lab: Hammersmith Medicines Research Ltd, Central Middlesex Hospital, London, UK. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. **GCP**: EC, Declaration of Helsinki, US, ICH.

*Aikens P, McBurney A, Bates T, Baldrey S, Low K & Shafait S (2003) Pharmacokinetics of ANS9801 and 9801-acid following a single dose by oral administration to healthy male volunteers. Report No. AJO 209/033072. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. **GLP**: UK, EC & OECD. **QA statement**: Yes.

Otabe A, Muraoka R & Narita T (2009a) ANS9801 Supplemental Report. Human Study ANSE-101. An open, dose escalating study to determine the safety. Tolerability, and pharmacokinetic profile of ANS9801 in healthy male volunteers. Sponsor: Ajinomoto Co Inc, Tokyo, Japan.

Experimental

Advantame (unspecified purity; Batch No. 010228; sourced from the Sponsor) was administered as a single oral dose in 150 mL water to groups of eight fasted male volunteers at 0.1, 0.25 or 0.5 mg/kg bw followed by a washout consisting of 2 x 75 mL of water. The dose selection was based on 2, 5, and 10-times the theoretical maximum daily intake in humans of 0.05 mg/kg bw. The mean age, height, weight and body mass index (BMI) were 24.1±5.2 years, 180.5±6.6 cm, 77.7±9.2 kg and 23.8±2.2 kg/m^2, respectively.

Plasma concentrations of Advantame and Advantame-acid were analysed in blood samples collected pre-dose, 5, 10, 15 and 30 min, and 1, 1.25, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 7, 8, 9, 10, 12, 24, 36, 48, 72, 96, 120, 144 and 168 h after dosing. The following pharmacokinetic parameters were analysed: C_{max}, T_{max}, K_{el}, AUC_t, AUC, AUC_{48} and $t_{1/2}$.
A physical examination was performed at screening (d -14 to -3), baseline (d -1), pre-dosing and d 2, 3 and 8. Heart rate and blood pressure were recorded at screening, baseline, pre-dosing, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 36, 48, 72, 96, 120, 144 and 168 h. ECG was conducted at screening, baseline, pre-dose and d 8. The following haematology and clinical chemistry parameters were analysed in blood samples collected at screening, baseline, 24, 36, 48, 72, 96, 120 and 168 h: Hb, RBC, WBC, differential WBC, Hct, MCV, MCH, MCHC, platelet count and PCV; albumin, ALP, AST, ALT, Cl, cholesterol, creatinine, gamma-GT, LDH, P, K, Na, total protein, urea and uric acid. Blood glucose was analysed pre-dose and 2 h post-dose. Urine was collected at screening, baseline, 24, 48 and 168 h post-dose and analysed for glucose, protein, bilirubin and ketones. Any adverse events were recorded.

Results

PHARMACOKINETICS

Pharmacokinetic parameters are summarised in Table 3.40 but are viewed as estimates only due to the underestimation of the Advantame-acid concentration. Advantame was below the limit of quantification (1 ng/mL) in all samples at the low-dose and in the majority of samples at the mid-dose. At the high-dose, no Advantame was detectable after 1 h. There was a proportional, dose-related increase in exposure to Advantame/Advantame-acid (C_{max} and AUC). The authors stated that the dose-related increase in $T_{1/2}$ for Advantame-acid was a result of its measurement over different time periods for each dose and therefore the values are not comparable. Based on the plasma concentrations of Advantame and Advantame-acid (at 0.5 mg/kg bw), it is estimated that >99% of the administered dose of Advantame is converted to Advantame-acid prior to absorption.

TABLE 3.40: PHARMACOKINETIC PARAMETERS IN MALE VOLUNTEERS

Parameter	0.1 mg/kg bw	0.25 mg/kg bw	0.5 mg/kg bw
Advantame			
C_{max} (ng/mL)	-	-	1.91±0.42
T_{max} (h)	-	-	0.5
AUC_t (ng.h/mL)	-	-	0.761±0.285
AUC_{48} (ng.h/mL)	-	-	0.875±0.301
Advantame-acid			
C_{max} (ng/mL)	11.8±3.6	24.5±7.5	46.8±18.3
T_{max} (h)	1.6	1.4	1.6
AUC_t (ng.h/mL)	73.6±33.3	237±74	459±142
AUC_{48} (ng.h/mL)	81.2±33.6	2143±72	458±146
AUC (ng.h/mL)	81.8±37.3	262±82	546±135
K_{el} (h^{-1})	0.1906±0.036	0.1149±0.0574	0.0635±0.0108
$T_{1/2}$ (h)	3.6	6.0	10.9

Results are expressed as the mean ± 1 SD

SAFETY

No subjects withdrew from the study. A total of 5 adverse events (described by the study authors as mild according to clinically validated international medical terminology) were recorded for three of the 24 subjects; one at the mid-dose (0.25 mg/kg bw) and two subjects at the top-dose (0.5 mg/kg bw). Such observations have a somewhat limited utility because of the absence of an untreated control group; it is therefore important to consider the consistency of any effects observed in other human studies.

In the mid-dose subject, a headache was reported seven days after a single dose and the study authors concluded that it was not related to treatment. FSANZ concurs with this conclusion because based on its elimination half-life ($T_{1/2}$) it seems very unlikely that any Advantame/Advantame-acid could be present in the body seven days after dosing – i.e. the headache is therefore unlikely to be associated with the presence of Advantame/Advantame-acid. There is also considerable doubt that the headache or dizziness reported by two individuals at the highest dose (0.5 mg/kg bw) is associated with exposure to Advantame/Advantame-acid because nobody else reported similar effects in any of the other human studies, including those involving larger groups of subjects (up to 36/group) over much longer periods of time (up to 12 weeks) at the same dose (Pirage et al 2006). Based on the lack of consistency across studies, the adverse events reported in this study are not considered treatment-related but to have arisen spontaneously.

There was no treatment-related effect on vital signs, ECG parameters, haematology, clinical chemistry or urinalysis parameters.

Conclusions

A single dose of Advantame up to 0.5 mg/kg bw was well tolerated.

Warrington S (2005) An open label study to investigate the adsorption, pharmacokinetics, metabolism and excretion of a single oral dose of ^{14}C-ANS9801 in healthy male volunteers. Study No. ANSE-102. Lab: Hammersmith Medicines Research Ltd, Central Middlesex Hospital, London, UK. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. **GCP**: EC, Declaration of Helsinki, US, ICH.

*Aikens P, Kane TJ, Milmoe S, McBurney A, Bates T & Baldrey S (2005e) Metabolism and Pharmacokinetic Report. An open label study to investigate the adsorption, pharmacokinetics, metabolism and excretion of a single oral dose of ^{14}C-ANS9801 in healthy male volunteers. Report No. AJO 210/042812. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Pharmaceuticals Europe Ltd on behalf of Ajinomoto Co Inc, Tokyo, Japan. **GLP**: UK, EC & OECD. **QA statement**: Yes.

Otabe A, Muraoka R & Narita T (2009b) ANS9801 Supplemental Report. Human Study ANSE-102. An open label study to investigate the adsorption, pharmacokinetics, metabolism and excretion of a single oral dose of ^{14}C-ANS9801 in healthy male volunteers. Sponsor: Ajinomoto Co Inc, Tokyo, Japan.

Experimental

Six healthy, fasted, male volunteers drank 150 mL of water containing 18.75 mg [^{14}C]-Advantame ($\geq$98% purity; Batch No. AJO186/57; sourced from the Sponsor) (~0.25 mg/kg bw) followed by a washout of 2 x 75 mL water. The dose selection was based on the results of the previous study (Warrington 2004). Additional water was ingested at 2 h post-dose. No control group was included because, similar to Warrington et al (2004), the study was designed to examine the metabolism of Advantame. Lunch was consumed at 4 h post-dose, with an evening meal given at 10 h post-dose. Only water was allowed to be consumed between meals. The mean age, height, weight and BMI were 49.5$\pm$8.83 years, 178.5$\pm$4.68 cm, 80.37$\pm$5.79 kg and 22.2-28.8 kg/m^2, respectively.

A full physical examination was conducted at screening, baseline and d 2 , 4 and 8. Heart rate and blood pressure were recorded at screening, baseline, pre-dosing and at 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 36, 48, 72, 96, 120, 144 and 168 h. ECGs were performed at screening, pre-dosing, 2 h and d 8. Blood was collected at screening, baseline, 24, 48, 168 h for analysis of the following haematology and clinical chemistry parameters: Hb, RBC, WBC, differential WBC, Hct, MCV, MCH, MCHC and platelet count; albumin, ALP, AST, ALT, Cl, cholesterol, creatinine, gamma-GT, LDH, P, K, Na, total protein, BUN and uric acid.

Midstream urine was collected at screening, baseline, 24, 48 and 168 h and analysed for the following: glucose, protein, bilirubin and ketones. Abnormal urine samples were subjected to a microscopic analysis.

Plasma concentrations of Advantame and Advantame-acid were analysed in blood samples collected pre-dose, 5, 10, 15, 30 and 45 min, and 1, 1.25, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 7, 8, 9, 10, 12, 24, 36, 48, 72, 96, 120, 144 and 168 h after dosing. Radioactivity was measured by LSC. Advantame and Advantame-acid were analysed in plasma by LC-MS/MS. The following pharmacokinetic parameters were analysed: C_{max}, T_{max}, K_{el}, AUC_{0-t}, $AUC_{0-\infty}$, AUC_{0-48} and $t_{1/2}$.

For the metabolite analysis, urine was collected at 12 h pre-dose, 0-1, 1-2, 2-3, 3-4, 4-8, 8-12, 12-24, 24-48, 48-72, 72-96, 96-120, 120-144 and 144-168 h. All faecal samples passed from 12 h prior to dosing until 168 h post dose were collected. Urine and faecal metabolites were analysed by HPLC with radiodetection.

Results

PHARMACOKINETICS

Pharmacokinetic parameters for total radioactivity and Advantame-acid are shown in Table 3.41, with the latter viewed as estimates only due. Radioactivity was absorbed relatively quickly, with the T_{max} = 1.25 h, but was eliminated more slowly, with the $T_{1/2}$ = 4 h.

TABLE 3.41: PHARMACOKINETIC PARAMETERS FOR MALE VOLUNTEERS

Parameter	Total radioactivity	Advantame-acid
C_{max} (ng/mL)	30.1±3.2	22.7
T_{max} (h)	1.25	1.75
AUC_t (ng.h/mL)	194±43	173±51
AUC_{48} (ng.h/mL)	223±55	183±50
AUC (ng.h/mL)	208±39	179±67
K_{el} (h^{-1})	0.177±0.027	0.122±0.0573
$T_{1/2}$ (h)	3.9	5.7

Results expressed as means ± 1 SD

METABOLISM

Total recovery of radioactivity in excreta was 91.7-101.7% of the administered radioactive dose, with the mean (±1SD) level of radioactivity in urine and faeces of 6.2% (range 3.6-11.8%) and 89.5% (range 87.2-92.8%), respectively. Based on the urinary level of radioactivity, GIT absorption is estimated to be ~6%. As it is not possible to estimate the proportion of the absorbed Advantame-acid excreted via the faeces because there are no IV dosing data, this estimate of GIT absorption is considered to be the minimum. In urine there were several peaks of radioactivity (2-3, 4-8 and 24-48 h), with the highest level occurring at the 24-48 h sampling interval; no radioactivity was detectable in any subjects after 120 h. The time of peak faecal radioactivity was generally at the 24-48 h interval, but was somewhat variable between subjects (0-24, 24-48, 48-72 or 72-96 h). Most radioactivity had been excreted via the faeces by 96 h, with low levels of radioactivity still detectable in 4 subjects during the last (144-168 h) sampling interval.

Metabolite profiles in excreta are summarised in Table 3.42. Advantame-acid and HU-1 (3-(3-hydroxy-4-methoxyphenyl)-1-propylamine) were the main metabolites in urine, with HF-1 (N-(3-(3-hydroxy-4-methoxyphenyl) also detected at approximately half their concentration. Advantame-acid accounted for approximately 40% of total urinary radioactivity, which suggests that absorbed Advantame-acid is extensively metabolised. In faeces, Advantame-acid and HF-1 were the only metabolites identified; HU-1 was not detected in faeces. It is

worth noting that the proportion of HF-1 in faeces was noticeably higher than that detected in dog faeces (<5%) (Aikens et al 2005b).

TABLE 3.42: METABOLITE PROFILES IN VOLUNTEERS

Metabolite	Urine	Faeces
HU-1	1.9±1.9	-
HF-1	1.0±0.6	30.0±12.0
Advantame-acid	2.3±0.6	52.0±13.0
Others	0.1±0.1	1.9±1.1

Results expressed as the mean % of the administered dose ± 1 SD

SAFETY

A total of 8 adverse events involving 5 subjects were reported. The majority (7) of these events were classified as mild, with one classified as moderate. Four subjects reported dental injury (broken teeth, lost dental filling) attributed to the consumption of hard, crusty bread rolls and the fifth experienced back pain. One of the subjects with dental injury also had insect bites (classified as mild), pain at the cannula site (where blood was withdrawn for testing) and rectal haemorrhage (classified as moderate), with the latter commencing six days after dosing and therefore not associated with Advantame treatment.

There was no treatment-related effect on vital signs, ECG parameters, haematology, clinical chemistry or urinalysis parameters.

Conclusions

A single dose of Advantame was well tolerated.

Krievins D (2005) A double-blind, randomised, placebo controlled, parallel-group study design in 24 normal healthy subjects to assess the tolerability of 4 weeks repeated administration of ANS9801. Study No. ANSE-103a. Lab: Stradins Clinical University Hospital, Riga, Latvia. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. **GCP**: EC, Declaration of Helsinki, US, ICH.

Baldrey S (2006) ANS9801 and ANS9801-Acid. Measurement of plasma concentrations and pharmacokinetics of ANS9801 and ANS9801-acid. Study No. AJO 0221/062130. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Pharmaceuticals Europe Ltd on behalf of Ajinomoto Co Inc, Tokyo, Japan. **GLP**: UK. **GCP**: UK, EC, ICH. **QA statement**: Yes.

Otabe A, Muraoka R & Narita T (2009c) ANS9801 Supplementary Analysis. Human Study ANSE-103a. Safety and tolerability assessment of multiple daily doses of ANS9801: Part 1. 4-week administration to normal human subjects. Sponsor: Ajinomoto Co Inc, Tokyo, Japan.

Experimental

Six subjects/sex/group were randomised to receive either 10 mg Advantame (0.375-0.5 mg/kg bw/day) (>99% purity, Lot No. 0279A; sourced from the Sponsor) or placebo (encapsulated cellulose) 3 times daily for 4 weeks. The subjects were blinded to the identity of their respective treatment. The dose selection was based on a previous study indicating that a dose of 0.5 mg/kg bw was well tolerated (Warrington 2004). Capsules were dispensed weekly, with subjects instructed to take a capsule with water at breakfast, lunch and dinner. Outside the examinations performed at the clinic, subjects self-reported any adverse events. The mean age and weight of the subjects were 34.5±11.85 years and 71.2±7.37 kg, respectively.

A full physical examination was conducted at screening, baseline, d 8, 15, 22 and 29, and

approximately one week after the final dose. Heart rate and blood pressure were also recorded at these times. ECGs were conducted at screening and d 29. Blood samples were collected at baseline, d 8, 15, 22 and 29 and approximately one week after the final dose for the analysis of the following haematology and clinical chemistry parameters: WBC, RBC, Hb, Hct, MCV, MCH, MCHC, differential WBC, platelet count; albumin, ALP, total bilirubin, BUN, Ca, Cl, cholesterol, C-peptide, HDL-cholesterol, LDL-cholesterol, creatinine, LDH, phosphate, K, SGOT, SGPT, Na, total protein, triglycerides and uric acid. Urine was collected at these same times and analysed for the following: pH, specific gravity, glucose, protein, bilirubin, ketones, occult blood and urobilinogen. Fasting blood glucose, HbA_{1C} and insulin were analysed at screening, baseline, d 8, 15, 22 and 29. On d 8 and 29, blood samples were collected immediately pre-dose and at 15, 30, 45, 60, 75, 90 and 120 min for the analysis of glucose and insulin. Plasma concentrations of Advantame and Advantame-acid were analysed in blood collected pre-dose and on d 8, 15, 22 and 29 by LC-MS/MS.

Results

PHARMACOKINETICS

Plasma concentrations of Advantame were below the LOQ (0.5 ng/mL) in all samples from the 12 subjects in the treated group. Plasma concentrations of Advantame-acid were highly variable; mean (± 1SD) concentrations at d 8, 15, 22 and 29 were 9.77±5.52, 10.3±7.5, 11.8±6.6 and 11.0±5.0 ng/mL, respectively. The consistency of these blood levels over the duration of the dosing period supplemented with regression analysis of individual data suggested that a steady state had been achieved. There was no evidence of accumulation.

SAFETY

Two subjects in the Advantame group experienced pruritus of the head, which was classified as mild. The study author considered that one of these cases of pruritis was possibly related to the test material but the other was unlikely to be related to the test material. On balance, this finding is not considered treatment-related. One control subject had a viral respiratory tract infection, also classified as mild.

There was no treatment-related effect on vital signs, ECG, haematology, clinical chemistry or urinalysis parameters. There was no difference between the treated and control group with regard to the oral glucose tolerance test or glucose/insulin profiles.

Conclusion

Advantame was well tolerated followed repeated oral dosing in males and females up to a dose of 0.5 mg/kg bw/day.

Pirage V (2006) Safety and tolerability assessment of multiple daily doses of ANS9801: Part 2 – A 12-week safety study of ANS9801 administered to subjects with type 2 diabetes. Study No. ANSE-103b. Lab: Stradins Clinical University Hospital, Riga, Latvia. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. **GLP**: UK. **QA Statement**: Yes. **GCP**: ICH.

Otabe A, Muraoka R & Narita T (2009d) ANS9801 Supplementary Analyses. Human Study ANSE-103b. Safety and tolerability assessment of multiple daily doses of ANS9801: Part 2. 12-week safety study of ANS9801 administered to subjects with type 2 diabetes. Sponsor: Ajinomoto Co Inc, Tokyo, Japan.

Experimental

Eighteen type 2 diabetics/sex/group were randomised to receive either 10 mg Advantame

(0.375-0.5 mg/kg bw/day) (>99% purity, Lot No. 0298A; sourced from the Sponsor) or placebo (encapsulated cellulose) 3 times daily for 12 weeks. The subjects were blinded to the identity of their respective treatment. The study was an extension of that described above (Krievins *et al* 2005). Capsules were dispensed monthly, with subjects instructed to take a capsule with water at breakfast, lunch and dinner. Outside of the examinations performed at the clinic, subjects self-reported any adverse events. The mean age and weight of the subjects were 60.1±7.30 years and 81.8±9.46 kg, respectively.

A full physical examination was conducted at screening (d -7), baseline (d 1), d 8, 29, 57 and 85, then approximately one week after the final dose. Heart rate and blood pressure were also recorded at these times. ECGs were conducted at screening and d 85.
Blood samples were collected at baseline, d 8, 29, 57 and 85 for the analysis of the following haematology and clinical chemistry parameters: WBC, RBC, Hb, Hct, MCV, MCH, MCHC, differential WBC, platelet count; albumin, ALP, total bilirubin, BUN, Ca, Cl, cholesterol, HDL-cholesterol, LDL-cholesterol, creatinine, LDH, phosphate, K, SGOT, SGPT, Na, total protein, triglycerides and uric acid. Urine was collected at these same times and analysed for the following: pH, specific gravity, glucose, protein, bilirubin, ketones, occult blood, urobilinogen and microscopy of spun deposits.

Fasting blood glucose and insulin were analysed at screening and d 8, 57 and 85. In a randomly selected subgroup of 12 males and 12 females, an oral glucose tolerance test was performed at screening, with samples taken at 15, 30, 45, 60, 75, 90 and 120 min post-dose. HbA_{1C} was analysed at screening (d -14) and d 8, 29, 57, 85 and 92.

Plasma concentrations of Advantame and Advantame-acid were analysed in blood collected pre-dose and on d 8, 29, 57 and 85, by LC-MS/MS.

Results

PHARMACOKINETICS

Advantame was not detectable in the plasma of any treated subject. Plasma concentrations of Advantame-acid were highly variable between subjects and ranged from 0-58.4, 0-46.4, 0-37.8 and 0-62.6 ng/mL at d 8, 29, 57 and 85, respectively. The mean concentrations at these sampling points were 16.2±11.9, 14.6±11.8, 12.5±9.7 and 13.6±14.2 ng/mL, respectively. These findings suggest that a steady state had been achieved over the dosing period and that there was no evidence of accumulation.

SAFETY

No subjects withdrew from the study. Nine subjects in the control group experienced a total of 10 adverse events, while 5 subjects in the treated group experienced a total of 9 adverse events. The majority of adverse events were classified as mild (11), with the remainder classified as moderate. The main adverse event was respiratory and/or urinary tract infection (6 subjects in the control group and 3 in the Advantame group), followed by gastrointestinal disorders (one subject in each group), asthenia (one subject in each group), dyslipiaemia (one control subject) and neuralgia (one control subject), all classified as slight. Of the 3 subjects with moderate adverse events, two were in the treated group (one with pneumonia; and the other with dyspepsia, flatulence and nausea) and the other was in the control (urinary tract infection). The authors considered that the occurrence of dyspepsia, flatulence and nausea of moderate severity in one Advantame subject was possibly treatment-related. However, given that this adverse event only occurred once in a single subject, this conclusion is questionable.

There was no treatment-related effect on vital signs, physical findings or ECG results. There

was no treatment-related effect on any haematology, clinical chemistry or urinalysis parameter. There was no difference between the treated and control groups with regard to the glucose/insulin profiles or HbA_{1C} concentration.

Conclusion

No adverse effects were evident when Advantame was orally-administered to Type-2 diabetics for 12 weeks including any effect on blood glucose. Advantame or Advantame-acid did not accumulate over 12-weeks period, with qualitative evidence that steady state had been achieved.

3.3 Discussion

Adequacy of the toxicological database

The toxicological database for Advantame is comprehensive and consists mainly of GLP and OECD test guideline compliant studies conducted in laboratory animals, and GCP studies conducted in humans. Data submitted in support of this Application are considered to be sufficiently comprehensive to define the hazard of Advantame and permit an ADI to be established.

Adsorption, distribution, metabolism and elimination

Advantame is relatively stable in simulated gastric fluid (SGF) but was rapidly (<5 min) and completely hydrolysed (i.e. de-esterified) to Advantame-acid in simulated intestinal fluid (SIF), with only limited hydrolysis occurring in the absence of pancreatin (Aikens et al 2002c). Advantame-acid was relatively stable in both SGF and SIF. These *in vitro* observations suggested that a substantial proportion of Advantame would be converted to Advantame-acid in the intestine and therefore only limited systemic exposure to the parent compound is likely. Indeed the level of GIT conversion estimated from *in vivo* toxicokinetic *data* was approximately 95% in mice (Chase 2002b) and rats (Chase 2002a; Chase et al 2004; Horne et al 2005), >99% in dogs (Barker et al 2002; Powell et al 2005; Powell & Scott 2006) and >97% in rabbits (Fulcher et al 2002). The absence of detectable concentrations of Advantame in urine supports the conclusion that there is limited systemic exposure to the parent compound following ingestion. Although only small doses have been given to humans (up to 0.5 mg/kg bw), GIT conversion is estimated to be >99% (Warrington 2004). To account for the variability in GIT conversion of Advantame to Advantame-acid, interspecies comparisons of key kinetic parameters are shown for total radioactivity (Table 3.43).

In non-fasted rats, absorption following oral gavage was relatively rapid, with maximum plasma concentrations reached in 15-45 min (Aikens et al 2004a). This was reasonably similar to fasted humans [T_{max} = 1.25 h (Aikens et al 2005c)], but contrasts with the T_{max} of 6-8 h in fasted dogs (Aikens et al 2005b). The elimination half-life from dog plasma was relatively long [~80 h; Aikens et al 2005b)]. In contrast, the apparent elimination half-life from human and rat plasma was much shorter [4 and 6-8 h, respectively (Warrington 2005 & Aikens et al 2004a, respectively)]. Intravenous dosing in rats revealed that Advantame/Advantame acid was excreted predominantly via the GI tract with only a quarter of the administered dose being found in urine. In contrast to rats, dogs appeared to reabsorb a significant proportion of the Advantame/Advantame acid excreted/secreted into the GI tract leading to much less being present in faeces (around half of an IV administered dose) and an increased amount in plasma and urine. The prolonged presence of radioactivity in plasma as shown by the very long apparent terminal half life in dogs is strongly suggestive of an enterohepatic circulation.

Based on pharmacokinetic data, bioavailability of radiolabelled Advantame-acid/metabolites was calculated to be 7-9% in rats (Aikens et al 2004a) and 8-15% in dogs (Aikens et al 2005b). There are no data to allow an estimation of bioavailability in rabbits. There was good agreement with estimates of bioavailability derived from the ratio of radioactivity in urine and faeces following IV dosing and the concentration of radioactivity in urine following oral dosing in rats and [8% (Aikens et al 2004a)] and dogs [8-14% (Aikens et al 2005b)]. While Advantame was not given to humans intravenously, based on levels of radioactivity in urine following oral dosing, bioavailability was estimated to be at least 6% (Aikens et al 2005e).

TABLE 3.43: INTERSPECIES COMPARISON OF SELECTED KINETIC PARAMETERS.

Parameter	Rat		Dog		Human
	Males	Females	Males	Females	Males
Dosing regimen (PO)	Non-fasted; gavage; *ad libitum* feeding		Fasted; gavage; food available from 4 h		Fasted; dosing via water; food eaten at 4 & 10 h
T_{max} (h)					
0.25 mg/kg bw	-	-	-	-	1.25[4]
5 mg/kg bw	0.25[1]	0.25[1]	7[3]	8[3]	-
150 mg/kg bw	0.75[1]	0.5[1]	6[3]	6[3]	-
$T_{1/2}$ (h)					
0.25 mg/kg bw	-	-	-	-	4[4]
5 mg/kg bw	7[1]	8[1]	86[3]	81[3]	-
150 mg/kg bw	6[1]	7[1]	74[3]	81[3]	-
F (%)					
5 mg/kg bw	8[1]	9[1]	15[3]	14[3]	-
150 mg/kg bw	7[1]	9[1]	9[3]	8[3]	-
Estimated bioavailability (%)	8[2,5]	-		8-14[3,5]	>6[4,6]

1 = Aikens et al 2004; 2 = Aikens et al 2005e; 3 = Aikens et al 2005b; 4 = Warrington 2005 & Aikens et al 2005e; 5 = Derived from the level of radioactivity in urine following oral dosing and the proportion of radioactivity detected in faeces following IV dosing; 6 = Derived from the level of radioactivity in urine following oral dosing

In rats and dogs, the kinetics of systemic exposure to Advantame-acid was variable but generally non-linear over repeated dosing regimens (typically less than the dose increment). A comparison of AUC_{∞} values for total radioactivity between rats and dogs at an equivalent single oral single gavage dose (5 mg/kg bw) (Aikens et al 2004a & 2005b, respectively) indicates that systemic exposure of dogs to radioactive metabolites is approximately 600-times higher than rats. Even by normalising the data for the difference in body surface area rather than bodyweight (ie. a 16-fold factor and linear kinetics assumed; Freireich et al 1966), dogs are exposed systemically to approximately 40-times more radioactive Advantame-acid metabolites than rats at an equivalent oral Advantame bodyweight-based dose. On this basis, dogs were the most heavily systemically exposed species investigated. Unfortunately, due to the absence of data, it is not possible to investigate whether a difference in pharmacokinetics was the cause of maternotoxicity in the main developmental toxicity study in rabbits.

The tissue distribution of radioactivity following a single dose of [^{14}C]-Advantame has been investigated in rats and dogs (Aikens et al 2002a & 2005b, respectively). Radioactivity was widely distributed in rats, with the highest concentrations detected in the contents of the GIT followed by the tissues of the GIT (i.e. stomach, small intestine and caecum) followed by the excretory organs (kidneys, liver and bladder). In rats, the majority of radioactivity was excreted by 24 h and there was no evidence of accumulation. Advantame or its metabolites did not cross the rat placenta. A similar pattern of tissue distribution occurred in dogs except that the elimination of radioactivity from plasma (and therefore tissue) was much slower. In dogs, only the bile and the large intestine wall had higher levels of radioactivity than plasma (~13- and 86-fold higher, respectively) (Aikens et al 2005b).

In rats, excretion of absorbed Advantame-acid was predominantly via the faeces (75%), with the remainder via the urine (Aikens et al 2005e). In dogs, the Advantame-acid was excreted

in approximately equal proportions in the urine and faeces (Aikens et al 2005a & b). In rats, excretion of radioactivity was completed by 48 h (most was excreted by 24 h) (Aikens et al 2002a, 2005d & 2005e), while in dogs, radioactivity was still detectable in faeces at the last sampling interval following oral (120 h) or IV dosing (168 h) contributing to incomplete recoveries (Aikens et al 2005b).

Metabolism of absorbed Advantame-acid following a single oral dose of Advantame is extensive in rats and dogs (Aikens et al 2004a and 2005b, respectively). An interspecies comparison of metabolites is presented in Table 3.44. A total of 4 faecal and 9 urinary metabolites have been identified in rats, dogs and humans. The main faecal metabolites included Advantame-acid, HF-1 (N-(3-(3-hydroxy-4-methoxyphenyl))propyl-L-aspartic acid) (dogs and humans) and RF-1 (demethylated Advantame-acid) and RF-2 (rats only). Additional urinary metabolites include HU-1 (3-[3-hydroxy-4-methoxyophenyl]-1-propylamine) and HF-1 (rats, dogs and humans). Unique urinary metabolites in rats include RU-1, RU-3 and Z1. Unique urinary metabolites in dogs include ZD-1, ZD-2 and D3. There are no data on the metabolites of Advantame generated in rabbits.

TABLE 3.44: ADVANTAME METABOLITES IN RATS, DOGS & HUMANS (% OF ADMINISTERED DOSE).

Metabolite	Rat[1]	Dog[2]	Human[3]
Faeces			
Advantame-acid	30/87 (5/150 mg/kg bw)	41/58 & 63/71 ♂/♀ (5 & 150 mg/kg bw)	52 (0.25 mg/kg bw)
HF-1	-	≤4.7% (5 & 150 mg/kg bw)	30 (0.25 mg/kg bw)
RF-2	12/0 (5/150 mg/kg bw)	-	-
RF-1	41/0 (5/150 mg/kg bw)	-	-
Zone 1[4]	-	0.7 & 0.4 (5 & 150 mg/kg bw)	-
Zone 2[4]	-	2.1 & 1.6 (5 & 150 mg/kg bw)	-
Urine			
Advantame-acid	≤0.6 (5 & 150 mg/kg bw)	3/3 & 2.4/0.5 ♂/♀ (5 & 150 mg/kg bw)	2.3 (0.25 mg/kg bw)
HU-1	≤0.3 (5 & 150 mg/kg bw)	0.3/0.3 & 0.2/0 ♂/♀ (5 & 150 mg/kg bw)	1.9 (0.25 mg/kg bw)
HF-1	0.3/0.1 (5 & 150 mg/kg bw)	1.3/1.5 & 0.3/0.1 ♂/♀ (5 & 150 mg/kg bw)	1 (0.25 mg/kg bw)
RU-2	≤0.3 (5 & 150 mg/kg bw)	-	-
Z-1[5]	≤0.2 (5 & 150 mg/kg bw)	-	-
RU-3[6]	≤0.3/0 (5 & 150 mg/kg bw)	-	-
ZD-1	-	0.3/0.2 & 0.1/0 ♂/♀ (5 & 150 mg/kg bw)	-
ZD-2	-	0.2/0.1 & 0.1/0 ♂/♀ (5 & 150 mg/kg bw)	-
D3	-	1.0/0.9 & 0.8/0.1 ♂/♀ (5 & 150 mg/kg bw)	-

Results expressed as the mean % of an administered radioactive dose of Advantame in parentheses; 1 = Aikens et al (2005e); 2 = Aikens et al (2005b); 3 = Warrington et al (2005); Aikens et al (2005e); 4 = Region on the chromatogram of unresolved radioactivity, which may represent undefined metabolites; 5 = indistinct zone of radioactivity containing at least one radioactive component; 6 = indistinct zone of radioactivity containing at least two radioactive components

Acute toxicity

The acute toxicity in rats was assessed as being very low, with an acute limit dose estimate of >5000 mg/kg bw (no deaths) (Blanchard & Clemson 2001). An acute gavage dose up to 1000 mg/kg bw had no effect on locomotor activity in rats (Williams & Adams 2001). A gavage dose of 1000 mg/kg bw Advantame decreased gastrointestinal motility in rats most likely due to the bulking effect of the compound (Williams et al 2001). In dogs, a single intraduodenal dose of Advantame up to 1000 mg/kg bw had no effect on cardiovascular or respiratory parameters (Jordan et al 2001).

Repeat-dose toxicity

Repeat-dose toxicity studies have been conducted in mice (13- & 104-weeks), rats (4-, 13- & 104-weeks) and dogs (4-, 13- & 52-weeks). No adverse effects clearly attributable to treatment occurred in mice, rats or dogs up to a maximum dietary concentration of 50000 ppm (up to approximately 9300, 6500 and 2500 mg/kg bw/day, respectively).

REDUCED BODYWEIGHT GAIN

In all but the 52-week dog study, reduced bodyweight gain occurred at the highest dose in one or both sexes. In some cases there was concomitant reduction in food conversion efficiency (4-week rat study; 13-week mouse study) but never any reduction in food consumption. Increased water consumption was recorded in the 4- and 13-week rat studies. The reduced bodyweight gain observed at the highest dose in dogs and rats is attributable to the relatively high (5% w/v) concentration of a non-caloric substance in the diet, which over repeated dosing regimens, results in a reduction in available energy/nutrients. On this basis, the reduced bodyweight gain is not considered a direct result of Advantame treatment.

DISCOLOURATION OF FAECES

A treatment-related but non-adverse observation in laboratory animals was discolouration of the faeces. In rats and dogs (but not mice), pale faeces were observed at the highest dietary concentration of 50000 ppm. Pale faeces were also observed in the preliminary developmental toxicity study in rabbits. As Advantame-acid is a white substance, it is likely that these observations are attributable to its presence in faeces. In the majority of rat studies (4-week, chronic, developmental and reproduction), green or purple faeces, and staining of the cage lining (due to contact with faecal material) were noted. The occurrence of green or purple faeces was not observed in dogs or rabbits.

HAEMATOLOGY

In rats and dogs, statistically significant differences in some haematology parameters (relative to the control group) were noted (summarised in Table 3.45). Each of these differences was evaluated in the context of the individual study and by looking for consistency across other studies. None of these differences was interpreted as being treatment-related based on the following considerations:

- Lack of consistency between sexes;
- Lack of a dose-response relationship;
- In dogs, some parameters were already significantly different to the control group prior to the commencement of dosing;
- The magnitude of the difference with the control group was within the variation over time for each group, including the control;
- In some cases, differences remained evident following the cessation of treatment;

- Where historical control data were available for age- and sexed matched animals, the values for treated groups were within the normal range; and
- Lack of consistency over the same duration of dosing in other studies.

TABLE 3.45: SUMMARY OF HAEMATOLOGY FINDINGS IN RATS AND DOGS

Study	Result	Interpretation
4-wk rat (Chase 2002a)	↓ Hct & Hb (♂) - all treated groups ↓ WBC & Hb (♀) – all treated groups	Not considered treatment-related: • No dose-response relationship. • No consistency between sexes (Hb & WBC) • No corroborative ↓ in RBC (♂) • Within the historical control range
13-wk rat (Chase et al 2004)	↓ Hct & Hb (♂ @ 50000 ppm & ♀ @ 15000 & 50000 ppm) ↓ RBC (♀ @ 50000 ppm) ↓ WBC (♂ @ 15000 & 50000 ppm) ↓ lymphocytes (♂ @ 15000 & 50000 ppm & ♀ @ 50000 ppm) No significant findings following recovery phase	Not considered treatment-related: • Within the historical control range • Similar differences not seen in the chronic study after 13-weeks of dietary exposure
104-wk rat (Horne et al 2005)	No significant findings during wk 13, 26, 39, 52, 78 or 104	-
4-wk dog (Barker et al 2002)	No significant findings	-
13-wk dog (Powell & Scott 2005)	↓ Hct, Hb & RBC @ 15000 & 50000 ppm (♀) ↓ Reticulocytes & WBC @ 50000 ppm (♀)	Not considered treatment-related: • Hb, WBC & reticulocytes already lower then control pre-treatment • For Hct & RBC - No dose-response - Similar differences not seen in males - Pattern and magnitude of variation over time consistent with other groups - Inconsistent with wk 13 findings in 52-week study
52-wk dog (Powell et al 2005)	↑ Hct and RBC during wk 13 (♂) at 50000 ppm ↑ Hct, Hb and reticulocytes (♀) during wk 52 at 10000 and 50000 ppm	Not considered treatment-related: • No consistency over time or between sexes • Inconsistent with 13-wk study • Hb and RBC (♂) already higher then control pre-treatment • Values for Hct, Hb and reticulocytes (♀) are consistent with values at earlier time points

Genotoxicity and carcinogenicity

Long-term rodent studies, including one commencing *in utero*, provided no evidence that Advantame was carcinogenic (Horne et al 2005; 2006a & b). Advantame was not mutagenic to bacteria or mammalian cells (May et al 2001; Clare et al 2002) or was not clastogenic in mice (Mehmood et al 2001).

Reproductive and developmental toxicity

There was no evidence of reproductive toxicity in rats up to a dose of 5900 mg/kg bw/day

(Willoughby 2004) and no evidence of teratogenicity in rabbits or rats up to doses of 2000 and approximately 5000 mg/kg bw/day, respectively (Fulcher et al 2002 & 2003; Willoughby 2003).

In the main rabbit developmental toxicity study (Fulcher et al 2003), the NOAEL for maternal and developmental toxicity was 500 mg/kg bw/day, based on the occurrence of clinical signs and mortalities (moribund sacrifices) in dams at and above 1000 mg/kg bw/day. Other results that were unusual compared to other tested laboratory species were green bladder contents and green staining of the surface of the kidneys in one mid-dose dam.

In the preliminary rabbit developmental toxicity study (Fulcher et al 2002), which was submitted as supplementary data by the Applicant during the first consultation round, green and/or purple and/or pink staining of the cage lining was observed for the majority of treated dams. Green and/or purple urine was observed directly in three mid-dose dams, while the one high-dose dam that was sacrificed in a moribund condition had green bladder contents and extensive green staining of the body surface. While these treatment-related abnormalities were consistent with observations made in the main study, there were no apparent treatment-related clinical signs or deaths.

Immunotoxicity

The immunotoxicity of Advantame was assessed as part of the 13-week rat study (Wing et al 2004) because reduced thymus weight occurred in high-dose males in the 4-week study (Chase et al 2002). This specific assessment for immunotoxicity did not demonstrate an effect of treatment. It is worth noting that thymus weight was unchanged in both the 13-week and 104-week rat studies. In mice, no difference in thymus weight occurred between the high-dose group and control in the 13-week study, while an *increase* in thymus weight occurred in the 104-week study that was not associated with any corresponding histopathology. In dogs, decreased thymus weight was noted in high-dose females in the 4-week study and in high-dose males in the 13-week study; no difference was observed in the 52-week study. As discussed in Section 3.2.5., the occurrence of reduced thymus weights (in dogs) was consistent with historical findings by the performing laboratory and was therefore not considered abnormal.

Human studies

Advantame at doses up to 0.5 mg/kg bw was well tolerated by normal and type-2 diabetic male and female volunteers following a single or repeated oral dose (via water or capsules) for up to 12 weeks (Krievins 2005; Pirage 2006; Warrington 2004 & 2005).

INTOLERANCE

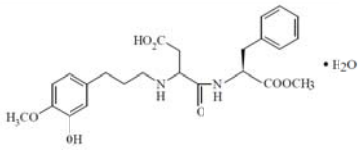
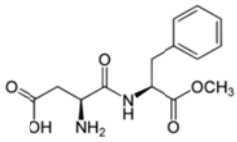
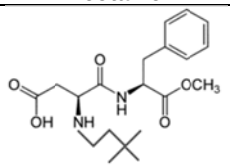
There are a number of findings that indicate intolerance reactions to Advantame would be unlikely:

- The aforementioned human studies detected no evidence of intolerance at doses up to 0.5 mg/kg/bw in a total of 110 subjects covering males and females, and type-2 diabetics.
- There was no effect on locomotor activity or behaviour in rats following a single oral dose up to 1000 mg/kg bw (Williams et al 2001; Williams & Adams 2001).
- There was no effect on motor activity, neuromuscular function or learning ability in rat offspring following repeated dosing up to ~6000 mg/kg bw/day (Willoughby 2004).
- In rats and dogs, Advantame or its metabolites did not accumulate in any tissue or organ (Aikens et al 2002a & b).

- There are no reports in the scientific literature of intolerance reactions to neotame, which is chemically and metabolically similar to Advantame (see below). While reports of intolerance to aspartame are available, this compound is considered a less useful surrogate than neotame because it is metabolised differently.

Comparison with other intense sweeteners

Advantame is an *N*-substituted (aspartic acid portion) derivative of aspartame that is similar in structure to neotame. On this basis it is worth briefly comparing the toxicological profile of these compounds in the context of the adverse effects observed for Advantame in rabbits. The chemical structure of Advantame, aspartame and neotame are given below:

Advantame	Aspartame	Neotame
		
ADI = 5 mg/kg bw/day (see below)	ADI = 40 mg/kg bw/day (JECFA 1980)	ADI = 2 mg/kg bw/day (JECFA 2004)
Based on a NOAEL of 500 mg/kg bw/day for deaths and clinical signs in rabbits & using a 100-fold safety factor.	Based on a NOAEL of 4000 mg/kg bw/day in rats & using a 100-fold safety factor.	Based on a NOAEL of 200 mg/kg bw/day for increased ALP in dogs & using a 100-fold safety factor.

The metabolism of Advantame, at least the first hydrolysis (i.e. de-esterification) step through non-specific esterases in the GIT that generates Advantame-acid, is shared with neotame. The hydrolysis reaction that produces de-esterified neotame also produces methanol in a 1:1 molar ratio (WHO 2004). The Applicant does not appear to have analysed the formation of methanol generated from the de-esterification of Advantame to Advantame-acid nor has its formation been shown in the proposed metabolic pathway in rats or dogs (see Sections 3.2.1.1 and 3.2.1.2). Based on the established chemistry of ester bond hydrolysis (that produces a carboxylic acid and ester alcohol) and that the identical methyl ester group on Advantame and neotame is hydrolysed, the formation of methanol from the ester hydrolysis of Advantame to Advantame-acid is considered probable. The generation of methanol would occur predominantly in the small intestine (where the majority of Advantame is hydrolysed), with the remainder then hydrolysed in the liver and plasma.

Humans are already exposed to methanol in the diet by virtue of its natural occurrence in a range of foods (including fruits and vegetables) and as a by-product of protein synthesis. For example, the concentration of methanol in fruit juices ranges from 12-640 mg/L (WHO 1997), with concentrations increasing during storage due to the breakdown of pectin (Lindinger et al 1997). The amount of methanol released during GIT hydrolysis of Advantame is much lower than exposure via these other sources[16]. The toxicological consequence of such dietary exposure to methanol has been assessed by various food regulatory authorities. For example, the UK's Committee on Toxicity (COT) has recently concluded that exposure to methanol in food, including that resulting from the consumption of aspartame, is unlikely to be harmful to human health (http://cot.food.gov.uk/pdfs/cotstatementmethanol201102.pdf).

[16] Theoretically, a complete equimolar conversion of Advantame to methanol at the maximum proposed level in beverages [4 mg/L or 0.008 mMoles/L (MW of Advantame = 476.52)], would yield 0.008 mMoles/L or 0.26 mg/L (MW of methanol = 32.06). This theoretical maximum would be about 50-2500 less methanol than naturally occurs in fruit juices. The amount of residual methanol present in commercial Advantame preparations is negligible (0.05%) and has been disregarded for this calculation.

The level of gastrointestinal absorption of neotame in rats, dogs and humans is similar to Advantame and elimination from plasma occurs more rapidly ($T_{1/2}$ = 1 h *versus* ~7 h in rats, respectively, for total radioactivity) (WHO 2004). For both compounds, the de-esterified parent is the main metabolite in excreta. Like the metabolism of Advantame-acid, metabolism of de-esterified neotame produces low concentrations of a number of minor metabolites (1-5%of an administered dose), including phenylalanine (WHO 2004). Based on the proposed metabolic pathways for Advantame in rats and dogs (see Sections 3.2.1.1 and 3.2.1.2, respectively) and the structural similarity with neotame, it is probable that some phenylalanine would be produced as a result of the metabolism of Advantame-acid to HU-1 (either directly or via HF-1). In establishing an ADI for neotame, the Joint WHO/FAO Expert Committee on Food Additives (JECFA) concluded that with regard to phenylketonuria, the formation of phenylalanine from the normal use of neotame "would not be significant in relation to this condition" (WHO 2004). This conclusion is also considered valid for Advantame. In conclusion, methanol and phenylalanine are natural dietary constituents and the very low levels generated from the normal metabolism of Advantame are considered to pose no public health and safety concerns.

For both Advantame and neotame, reduced bodyweight gain occurred at high doses in laboratory animals. For neotame only, this was coincident with reduced food consumption that was attributable to decreased palatability at dietary concentrations >150 ppm (WHO 2004). Consistent with observations for Advantame in rats and dogs, pale or discoloured faeces were observed in dogs (but not rats) dosed with neotame, due to unabsorbed neotame/de-esterified neotame in the GIT (WHO 2004). None of these treatment-related findings for neotame were considered toxicologically significant by JECFA. The pivotal endpoint for neotame and the basis for the current ADI is elevated ALP in dogs. There was no evidence that Advantame caused a similar increase in ALP. With regard to the toxicity of Advantame to rabbits, studies conducted on neotame tested lower doses (up to 1000 mg/kg bw/day) than Advantame (up to 2000 mg/kg bw/day) and therefore a direct dose comparison is not possible.

ADI considerations

No adverse effects occurred in rats or dogs following repeated dietary exposure up to doses of approximately 6500 and 2500 mg/kg bw/day, respectively. In humans, no adverse effects occurred following repeated oral doses (via capsules) up to 0.5 mg/kg bw/day for 12 weeks.

The only study in the extensive toxicological database for Advantame where treatment-related, adverse effects were observed was the main rabbit developmental study by Fulcher et al (2003). The NOAEL was 500 mg/kg bw/day, based on the occurrence of deaths (early sacrifices) and clinical signs in dams at and above 1000 mg/kg bw/day. In the preliminary developmental toxicity study, which tested the same doses, no apparent treatment-related adverse effects occurred (Fulcher et al 2002). However, in both rabbit studies, green or purple staining of the urine was evident; this is noteworthy because similar urinary staining was not directly observed in rats or dogs. The identity of the compounds (most likely Advantame-acid metabolites) responsible for the green or purple colouration of rabbit urine has not been determined.

The direct observation of green or purple urine, bladder contents, external body surfaces (due to soiling from urine) and in some cases internal surfaces (such as the kidneys) appears to be unique to rabbits and suggests systemic exposure to compounds not present systemically in rats or dogs, or present at much lower levels. Given these observations, and the absence of pharmacokinetic and metabolism data for rabbits, the maternotoxicity in rabbits cannot be disregarded as relevant to humans without further experimental investigation.

FSANZ maintains that the adverse, treatment-related findings observed in rabbits cannot be discounted without additional data to show that the findings are not toxicologically relevant. On the basis that there are inadequate data available to discount the rabbit findings as being relevant to this risk assessment, the ADI for Advantame is based on the NOAEL of 500 mg/kg bw/day in the main rabbit development study. The application of a 100-fold uncertainty factor yields an ADI of 5 mg/kg bw/day.

3.4 Conclusion

Based on an independent assessment of the extensive toxicological database for Advantame, no public health and safety concerns were identified from its proposed use as an intense sweetener.

The ADI for Advantame is 5 mg/kg bw/day.

4. Dietary Exposure Assessment

4.1 Approach to estimating dietary exposure to Advantame

Dietary exposure assessments (DEAs) require data on concentrations of the chemical of interest in food and food consumption data. The approach for this DEA was to use chemical concentration data as proposed by the Applicant combined with food consumption data available from the most recent Australian and New Zealand national nutrition surveys. The dietary exposure assessment was conducted using FSANZ's dietary modelling computer program, DIAMOND.

A summary of the FSANZ approach to conducting dietary exposure assessments is at Appendix 4. A detailed discussion of the FSANZ methodology and approach to conducting dietary exposure assessments is set out in the *Principles and Practices of Dietary Exposure Assessment for Food Regulatory Purposes* (FSANZ 2009a).

4.1.1 Proposed foods and concentration data used

The Applicant has provided the proposed food uses and use levels for Advantame in Australia and New Zealand, as set out in Table 2.4, Section 2 of this Report.

For the purposes of estimating dietary exposure to Advantame, the food categories proposed by the Applicant were assigned to DIAMOND food classification codes. These codes are based on the Australia New Zealand Food Classification System (ANZFCS) used in Schedule 1 to Standard 1.3.1 – Food Additives, of the Code. Foods for which permission to add Advantame has been requested, corresponding ANZFCS food groups included in the DEA and the proposed concentration of Advantame for each food group are set out in Table 4.1.

In order to ensure a conservative estimate of dietary exposure, where permission to add Advantame at certain concentrations to specific foods was proposed, the whole group of foods to which the specific food belongs was included in the model and assumed to contain Advantame at that concentration. For example, while permission was requested to add Advantame to 'powdered flavoured milk drinks' the category of 'liquid milk products and flavoured liquid milks' has been included in the assessment. Overall this has resulted in a much broader range of foods being included in the assessment than has been requested and consequently a highly conservative estimate of dietary exposure has been made.

TABLE 4.1: REQUESTED FOOD USES AND CORRESPONDING DIAMOND FOOD CLASSIFICATION AND ADVANTAME CONCENTRATION USED FOR DIETARY EXPOSURE ASSESSMENT.

Requested Advantame food uses	DIAMOND Food Code (1995 and 1997 NNS)	DIAMOND Food Code (2007 NNS)	DIAMOND food group	Advantame concentration (mg/kg)*
Powdered milk and non-milk based meal replacements and protein drinks	13.3	13.3	Formula meal replacements and supplementary foods	5
	13.4	13.4	Formulated supplementary sports foods	5
Instant teas	14.1.5.1	14.1.5.3	Tea	3
Instant coffee drinks	14.1.5.4	14.1.5.1	Coffee beverage	2
	14.1.5.5	14.1.5.1	Decaffeinated coffee beverage	2
	14.1.5.6	14.1.8	Coffee substitutes beverage	2
	14.1.5.7	14.1.5.1	Coffee-based mixes beverage	2
Powdered flavoured milk and milk drinks	1.1.2	1.1.2	Liquid milk products and flavoured liquid milk	5
	20.1.1	20.1.1	Beverages made up from beverage flavouring	5
Powdered fruit flavoured drinks	14.1.3.4	14.1.3.1.3	Cordial	4
	14.1.3.5	14.4.3.1.2	Electrolyte/sports drinks and electrolyte drink base	4
Table top sugar substitutes (powder, liquid and tablets)	11.4	11.4	Table top sweeteners	450

*The proposed maximum use level of Advantame in specified foods was rounded up to the nearest whole number for use in the DEA. Advantame concentrations proposed by the Applicant are set out in Table 2.4.

4.1.2 Consumption data used

Food additive permissions contained in the Code apply to food produced or sold in both Australia and New Zealand. Therefore this dietary exposure assessment has been conducted for both countries.

Food consumption data used for these assessments include:

- 1995 Australian National Nutrition Survey (1995 AusNNS), one 24 hour food recall survey covering 13,858 Australians aged 2 years and above
- 1997 New Zealand National Nutrition Survey (1997 NZNNS), one 24 hour food recall survey covering 4,636 New Zealanders aged 15 years and above
- 2007 Australian National Children's Nutrition and Physical Activity Survey (also known as 'Kids Eat Kids Play') (2007 AusNNS) two non-consecutive 24 hour food recall surveys covering 4,487 Australian children aged 2-16 years.

The design of these surveys varies somewhat and key attributes of each, including survey limitations, are set out in Appendix 4.

The hazard identification and characterisation (Section 3) did not identify any population sub-groups for which there were specific safety considerations in relation to Advantame. Therefore, the population groups selected for the DEA were matched with the most recent food consumption data available. The sub groups included in this assessment were:

- Australians aged 2-6 years, 7-16 years, and 17 years and above
- New Zealand population aged 15 years and above.

Children aged 2-6 years are included as they have the highest food intake on a per kilogram body weight basis, due to their lower body weights and proportionally higher energy needs as they are growing and developing (FSANZ 2009a, Section 4.3).

4.1.3 Assumptions and limitations of the Dietary Exposure Assessment

Assumptions made in the dietary exposure assessment include:

- where permission for Advantame was given to a food classification code, all foods in that group contained Advantame
- unless otherwise specified, all the foods within the group contained Advantame at the level specified in 4.1, above
- where a food was not included in the exposure assessment, it was assumed to contain a zero concentration of Advantame
- where a food has a specified Advantame concentration, this concentration is carried over to mixed foods where the food has been used as an ingredient
- there are no reductions in Advantame concentrations from food preparation or due to cooking
- there is no contribution to Advantame exposure through the use of medicines.

These assumptions are likely to lead to a considerable over-estimate for Advantame dietary exposure.

In addition to the specific assumptions made in relation to this DEA, there are a number of limitations associated with the dietary survey data upon which the DEA is based. A discussion of these limitations is included in Section 6 of the *Principles and Practices of Dietary Exposure Assessment for Food Regulatory Purposes* (FSANZ 2009a).

4.2 Estimated dietary exposure to Advantame

DEA results for Advantame were calculated for 'consumers' only, that is, those people in each NNS who reported consuming food that was assumed to contain the additive. Population statistics (mean and 90th percentile estimated exposure, on a milligrams per day basis) for each population group assessed were derived from each individual's ranked exposures. Intakes were then derived on a per kilogram body weight basis, using each individual's body weight, and reported as a proportion of the ADI. Major dietary contributors to the total intake of Advantame were also calculated for each population group.

4.2.1 Dietary exposure estimates for each population group assessed

Estimated mean and 90th percentile dietary exposure to Advantame were at or below 5 mg/day for all population groups assessed, with Australian adults aged 17 years and above estimated as having the highest daily dietary exposure (2.7 and 5 mg/day for mean and 90th percentile estimated exposure, respectively) followed by the New Zealand population aged 15 years and above (2.2 and 4.2 mg/day for mean and 90th percentile estimated exposure respectively).

When compared with the reference health standard of 5 mg/kg bw/day proposed by FSANZ,

estimated mean and 90th percentile dietary exposures were less than 3% of the ADI for all population groups assessed. Estimated mean and the 90th percentile exposures for Advantame as a percentage of the ADI were higher for children compared to adults, which is expected as a result of their lower body weight ratio compared to food consumption.

These results are summarised in Figures 4.1 and 4.2 below, with detailed results set out in Tables A5.1 and A5.2 of Appendix 5.

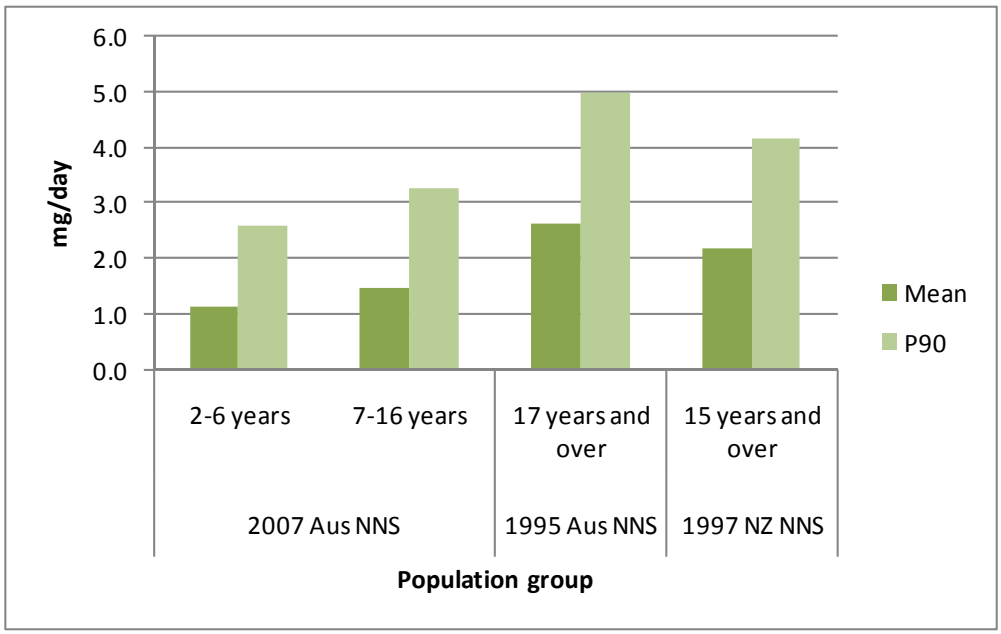


FIGURE 4.1: MEAN AND 90TH PERCENTILE ESTIMATED DIETARY EXPOSURE TO ADVANTAME (MG/DAY) FOR ALL POPULATION GROUPS ASSESSED

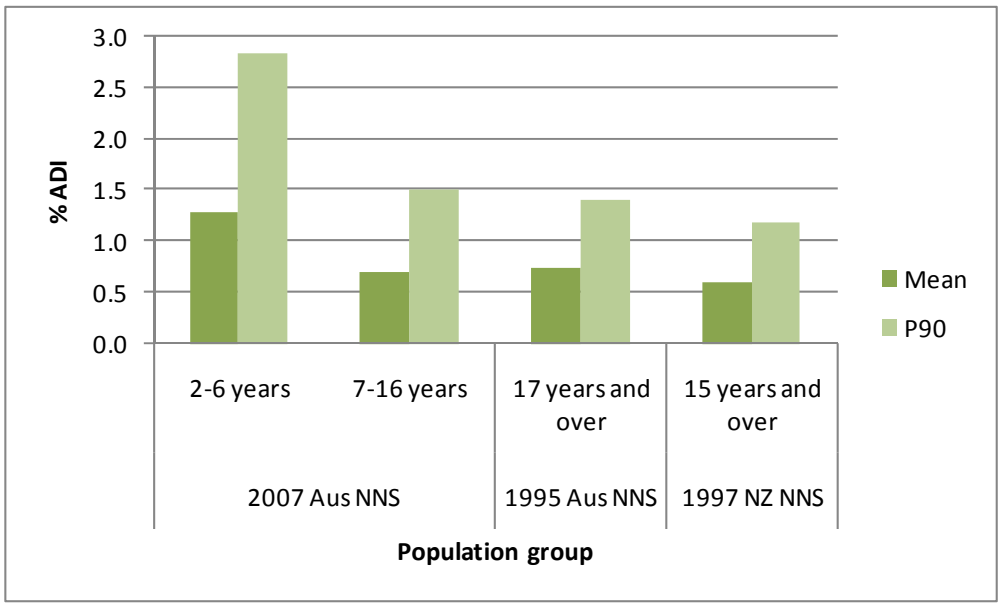


FIGURE 4.2: MEAN AND 90TH PERCENTILE ESTIMATED DIETARY EXPOSURE AS A PROPORTION OF THE ACCEPTABLE DAILY INTAKE (5 MG/KG BW/DAY) FOR ALL POPULATION GROUPS ASSESSED

4.2.2 Major foods contributing to Advantame exposure

Major foods contributing to Advantame dietary exposure were calculated from consumers' mean intake of foods proposed to contain the additive. Given the inclusion of much broader food categories in the model than were requested by the Applicant, these data should be interpreted with caution. For example, the Applicant requested permission to add Advantame to instant teas; however, for the purposes of the DEA all tea was included in the assessment. This is likely to have lead to a substantial overestimation of the contribution of tea to total Advantame exposure as the majority of tea consumed is not made from instant tea.

For the purposes of calculating the major foods predicted to contribute to total Advantame exposure, similar food groups set out in Table 4.1 were combined. For example all food codes relating to coffee were combined under the group 'coffee beverages'.

For Australian children aged 2-6 years and 7-16 years the major predicted contributors to total Advantame exposure were liquid milk products and flavoured liquid milk, cordials, electrolyte and sports drinks, formulated meals and sports foods and beverages made up from flavourings. For the Australian adult population aged 17 years and above and New Zealand population aged 15 years and above, the major contributors to total Advantame exposure was predicted to be tea, coffee beverages and cordials, electrolyte and sports drinks.

The major contributors (≥5%) are shown in Figure 4.3 for Australians aged 2-6 years, Figure 4.4 for Australians aged 7-16 years, Figure 4.5 for Australians aged 17 years and above and Figure 4.6 for New Zealanders aged 15 years and above. A detailed list of all the food groups and their contributions to Advantame dietary exposure can be found in Table A5.3 of Appendix 5.

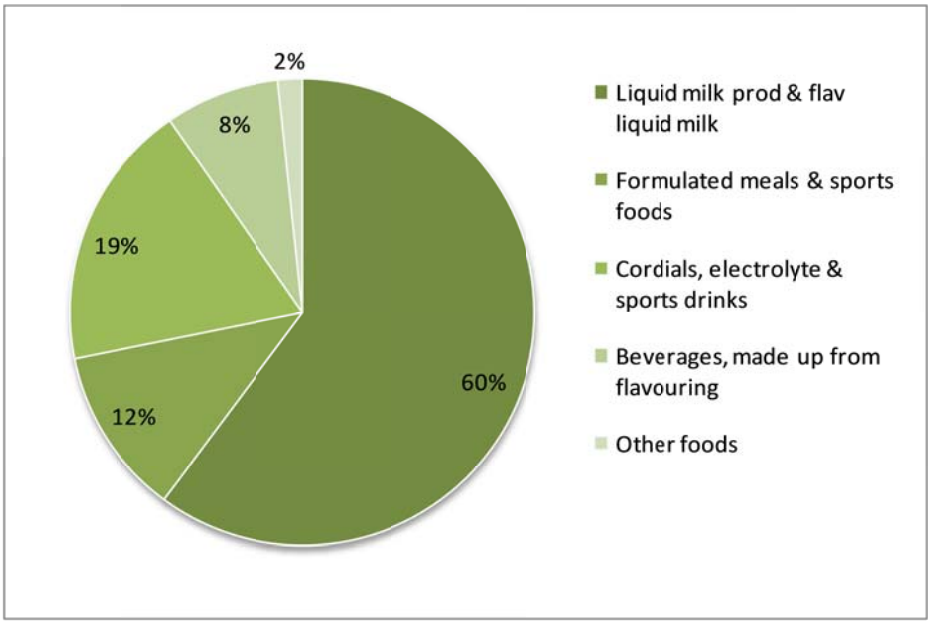


FIGURE 4.3: MAJOR CONTRIBUTORS TO ESTIMATED ADVANTAME DIETARY EXPOSURE FOR AUSTRALIAN CHILDREN AGED 2-6 YEARS

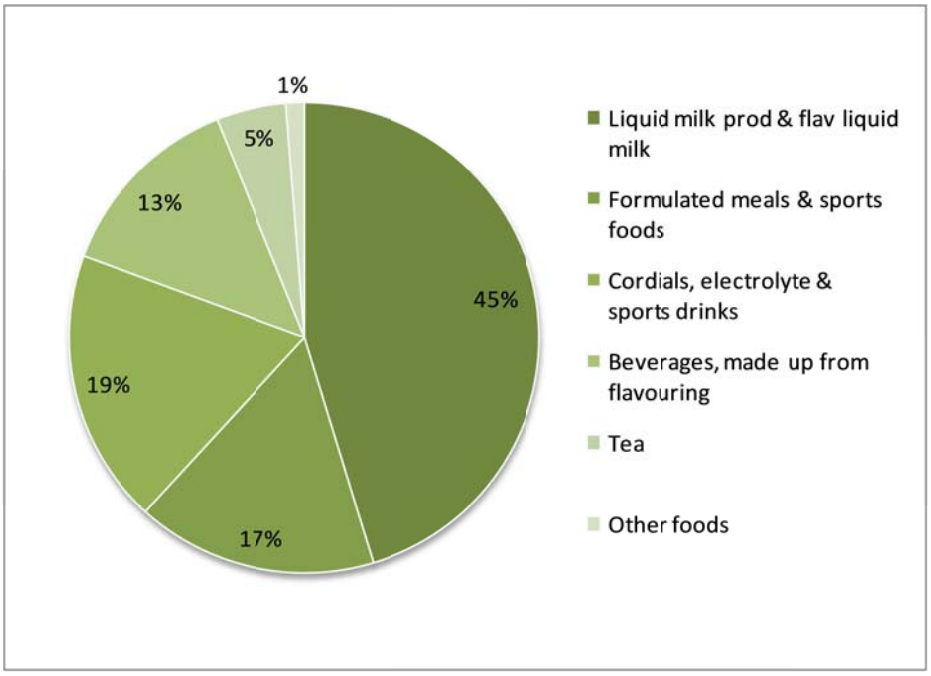


FIGURE 4.4: MAJOR CONTRIBUTORS TO ESTIMATED ADVANTAME DIETARY EXPOSURE FOR AUSTRALIAN CHILDREN AGED 7-16 YEARS

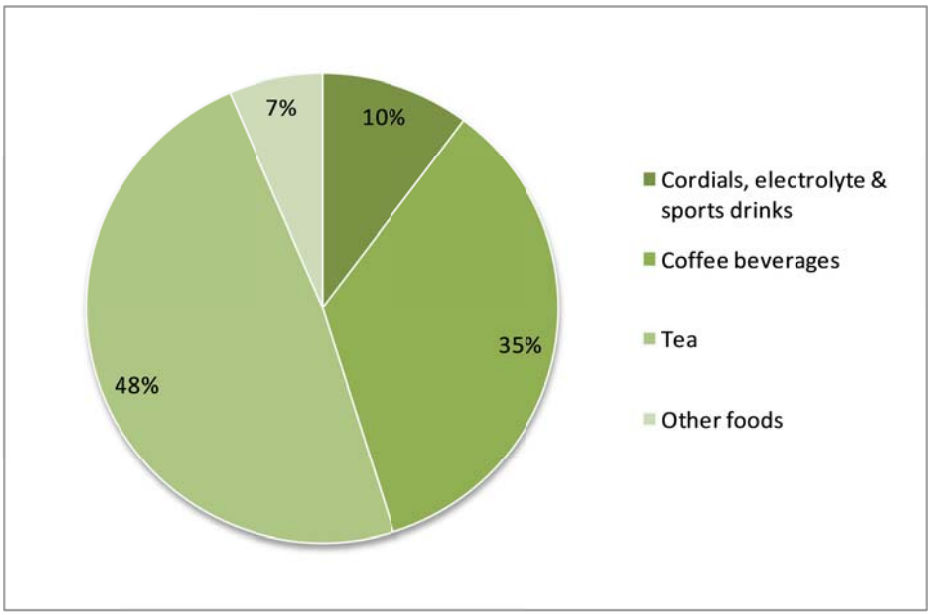


FIGURE 4.5: MAJOR CONTRIBUTORS TO ESTIMATED ADVANTAME DIETARY EXPOSURE FOR THE AUSTRALIAN POPULATION AGED 17 YEARS AND OVER

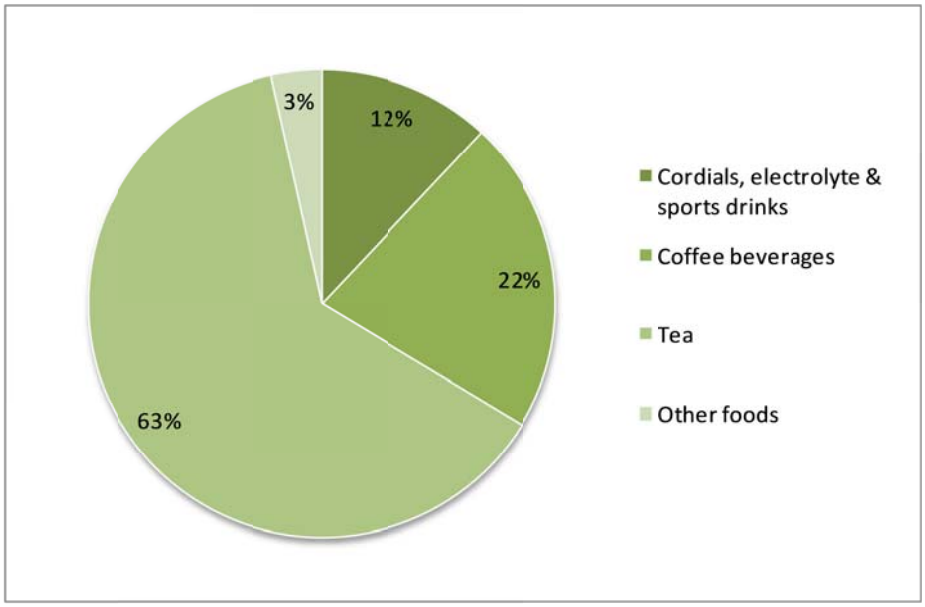


FIGURE 4.6: MAJOR CONTRIBUTORS TO ESTIMATED ADVANTAME DIETARY EXPOSURE FOR THE NEW ZEALAND POPULATION AGED 15 YEARS AND OVER

4.4 Conclusion

The DEA for Advantame, modelled to include broad food groups based on proposed specific food uses and maximum concentrations requested by the Applicant, indicated that estimated dietary exposures to Advantame were very low. Both mean and 90th percentile estimated dietary exposures for all population groups assessed were less than 3% of the reference health standard of 5 mg/kg bw/day proposed by FSANZ.

The DEA indicated that, for Australian children aged 2-6 years and 7-16 years, the major contributors to estimated Advantame dietary exposure would be liquid milk products and flavoured liquid milks, formulated meals and sports foods, and cordials, electrolyte and sports drinks. For older age groups, the major contributors to estimated Advantame dietary exposure were tea and coffee beverages.

5. Risk Characterisation

Comparisons of the dietary exposure to Advantame with the ADI of 5 mg/kg bw indicated that for all groups of Australian and New Zealand consumers assessed (including children), estimated dietary exposures were well below this safe level of exposure.

The estimated mean dietary exposures for consumers of Advantame correspond to 1.3% of the ADI for Australians aged 2-6 years, 0.7% of the ADI for Australians aged 7-16 years, 0.7% of the ADI for Australians aged 17 years and above, and 0.6% of the ADI for New Zealanders aged 15 years and above.

The estimated 90th percentile dietary exposures for consumers of Advantame correspond to 2.8% of the ADI for Australians aged 2-6 years, 1.5% of the ADI for Australians aged 7-16 years, 1.4% of the ADI for Australians aged 17 years and above, and 1.2% of the ADI for New Zealanders aged 15 years and above.

These comparisons raise no public health and safety issues for the addition of Advantame at the proposed levels of use.

6. Conclusions

- The proposed use of Advantame as an intense sweetener is technologically justified.
- The toxicity of Advantame has been well characterised based on an extensive database. The ADI for Advantame is set at 5 mg/kg bw/day.
- For all groups of Australian and New Zealand consumers assessed (including children), estimated dietary exposures were well below the ADI.
- There are no public health and safety issues associated with the proposed addition of Advantame to food.

References

Aikens PJ, Kirkpatrick D, Pluthero MG & Kane TJ (2002a) ANS9801. Tissue distribution in the male rat. Report No. AJO 181/013583. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. *Unpublished*.

Aikens PJ, Kirkpatrick D, Pluthero MG & Kane TJ (2002b) ANS9801. Tissue distribution in the male dog. Report No. AJO 191/022818. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. *Unpublished*.

Aikens PJ, Kirkpatrick D, Pluthero MG & Wallbank K (2002c) ^{14}C-ANS9801 and ^{14}C-ANS9801-acid: Stability in simulated gastric and intestinal fluid. Report No. AJO 173/013290. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. *Unpublished*.

Aikens P, McBurney A, Bates T, Baldrey S, Low K & Shafait S (2003) Pharmacokinetics of ANS9801 and ANS9801-acid following a single dose by oral administration to healthy male volunteers. Report No. AJO 209/033072. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Aikens PJ & Hickson JA (2004) ^{14}C-ANS9801 and ^{14}C-ANS9801-acid. Studies of plasma protein binding *in vitro* (rat, dog and human). Report No. AJO 213/033887. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. *Unpublished*.

Aikens PJ, Kane TJ & Houchen T (2004) ANS9801. Determination of the distribution in rats by whole-body autoradiography. Report No. AJO 217/042246. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. *Unpublished*.

Aikens PJ, Kirkpatrick D, Pluthero MG, Kane TJ, Baldrey SF, Flack I, Shafait S & Holding JD (2004) ANS9801. Pharmacokinetics of single doses in the rat after oral and intravenous administration. Report No. AJO 184/034042. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. *Unpublished*.

Aikens PJ, Kane TJ & Pluthero MG (2005a) ANS9801. Metabolism in the rat preliminary investigations. Report No. AJO 172/014349. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. *Unpublished*.

Aikens PJ, Kirkpatrick D, Kane TJ, Swan GJ & Milmoe S (2005b) ANS9801. Metabolism in the rat. Report No. AJO 194/042944. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. *Unpublished*.

Aikens PJ, Kirkpatrick D, Kane TJ & Holding JD (2005c) ANS9801. Metabolism in the dog. Preliminary investigations. Report No. AJO 180/013772. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. *Unpublished*.

Aikens PJ, Kirkpatrick D, Kane TJ, Swan GJ, Milmoe S, Baldrey SF & McBurney (2005d) ANS9801. Metabolism and pharmacokinetics in the dog. Report No. AJO 193/042943. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. *Unpublished*.

Aikens P, Kane TJ, Milmoe S, McBurney A, Bates T & Baldrey S (2005e) Metabolism and Pharmacokinetic Report. An open label study to investigate the adsorption, pharmacokinetics, metabolism and excretion of a single oral dose of ^{14}C-ANS9801 in healthy male volunteers. Report No. AJO 210/042812. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Pharmaceuticals Europe Ltd on behalf of Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Aikens P, Kane TJ & Milmoe S (2005f) ^{14}C-ANS9801. Comparison of metabolite profiles in rats, dogs and humans. Report No. AJO 218/042955. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. *Unpublished*.

Aleman CL, Noa M, Mas I, Rodeiro R, Mesa R, Menendez R, Gamez R & Hernandez C (2000) Reference data for the principal physiological indicators in three species of laboratory animals. Laboratory Animals **34**: 379-385.

Amino Y, Mori K, Tomiyama Y, Sakata H & Fujieda T (2008) Development of new, low calorie sweetener: new Aspartame derivative. In "Sweetness and sweetners: biology, chemistry, and psychophysics". Ed: Weerasinghe, DK and Dubois GE. Pub. ACS, Washington DC, p 463-480.

Baldrey SF, Phelan MR, McBurney A & Holding J (2001) Pharmacokinetic report. Pharmacokinetics of ANS 9801 and ANS 9801-acid following a maximum tolerated dosage study by dietary administration to beagle dogs. Report No. AJO 156/012585. *Unpublished*.

Baldrey SF, Flack I & Carr M (2002a) Toxicokinetic report. ANS9801. Toxicity study by dietary administration to CD-1 mice for 13 weeks. Report No. AJO 174/014348. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Baldrey SF, Phelan MR, McBurney & Holding J (2002b) Pharmacokinetics of ANS9801 and ANS9801 following a maximum tolerated dosage study by dietary administration to Han Wistar rats. Report No. AJO 151/012004. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Baldrey SF, Flack I, Phelan M, Williams M & Boussalt A (2002c) ANS9801 and ANS9801-Acid. Validation of a LC-MS/MS bioanalytical method for the measurement of ANS9801 and ANS9801-Acid in rat plasma. Report No. AJO 147/012240. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. *Unpublished*.

Baldrey SF, Flack I, Phelan M, Williams M & Boussalt A (2002d) ANS9801 and ANS9801-Acid. Partial validation of a LC-MS/MS bioanalytical method for the measurement of ANS9801 and ANS9801-Acid in dog plasma. Report No. AJO 153/012241. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. *Unpublished*.

Baldrey SF, Flack I & Phelan M (2002e) ANS9801 and ANS9801-Acid. Partial validation of a LC-MS/MS bioanalytical method for the measurement of ANS9801 and ANS9801-Acid in mouse plasma. Report No. AJO 169/014336. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. *Unpublished*.

Baldrey SF, Flack I & Phelan M (2002f) ANS9801 and ANS9801-Acid. Partial validation of a LC-MS/MS bioanalytical method for the measurement of ANS9801 and ANS9801-Acid in rabbit plasma. Report No. AJO 170/014335. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. *Unpublished*.

Baldrey SF, Phelan MR, Holding J, Carr MJ & Cook SC (2002g) Toxicokinetic Report. ANS9801: Preliminary embryo-fetal toxicity in the rabbit by gavage administration. Report No. AJO 183/010158. *Unpublished*.

Baldrey SF, Phelan MR, Holding J, Carr MJ & Cook SC (2004) Toxicokinetic report. ANS9801. Toxicity study by dietary administration to Han Wistar rats for 13 weeks followed by a 4 week recovery period. Report No. AJO 176/014075. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Baldrey SF, Flack I, McBurney A & Carr MJ (2005) Pharmacokinetics of ANS 9801 and ANS 9801-acid following dietary administration to beagle dogs for 13 weeks followed by a 4-week recovery period. Report No. AJO 179/014664. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Baldrey S (2006) ANS9801 and ANS9801-Acid. Measurement of plasma concentrations and pharmacokinetics of ANS9801 and ANS9801-acid. Study No. AJO 0221/062130. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Pharmaceuticals Europe Ltd on behalf of Ajinomoto Co Inc, Tokyo, Japan. *Unpupblished*.

Barker MH et al (2002) ANS9801. Toxicity study by dietary administration to beagle dogs for 4 weeks. Report No. AJO 156/012585. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Bates T, Baldrey S, Shafait S & Low K (2005) ANS9801 and ANS9801-Acid. Validation of a LC-MS/MS bioanalytical method for the measurement of ANS9801 and ANS9801-Acid in human plasma. Report No. AJO 206/032893. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. *Unpublished*.

Blanchard EL & Clemson AD (2001) ANS9801: Acute oral toxicity to the rat (acute toxic class method). Report No. AJO 155/012600/AC. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Charles River Laboratories (2002) Postnatal growth, development and behavioral/functional evaluation in Crl:CD (SD)IGS BR rats. Available online at http://www.criver.com/SiteCollectionDocuments/rm_rm_r_postnatal_evaluation_crlcd_sd_igs_br_rats.pdf

Charles River Laboratories (2003) Spontaneous neoplasms and survival in Wistar Han rats: compilation of control group data. Available online at http://www.criver.com/SiteCollectionDocuments/rm_rm_r_survival_wistar_han_rats_compilation_data.pdf

Charles River Laboratories (2005) Spontaneous neoplastic lesions in the Crl:CD-1(ICR) mouse in control groups from 18 month to 2 year studies. Available online at http://www.criver.com/SiteCollectionDocuments/rm_rm_r_lesions_crlcd_1_icr_mouse.pdf

Charles River Laboratories (2008) Clinical laboratory parameters for Crl:WI (Han). Available online at http://www.criver.com/SiteCollectionDocuments/rm_rm_r_Wistar_Han_clin_lab_parameters_08.pdf

Chase K (2002a) ANS9801. Toxicity study by dietary administration to CD-1 mice for 13 weeks. Report No. AJO 174/014348. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Chase KC (2002b) ANS9801: Preliminary toxicity study by dietary administration to Han Wistar rats for 4 weeks. Report No. AJO 151/012004. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Chase K et al (2004) ANS9801. Toxicity study by dietary administration to Han Wistar rats for 13 weeks followed by a 4 week recovery period. Report No. AJO 176/014075. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Clare MG, Kernahan J & Clemson AD (2002) ANS9801. Mammalian cell mutation assay. Report No. AJO 159/013035. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Elmore SA (2006) Histopathology of the lymph nodes. Toxicologic Pathology **34**: 425-454.

Freireich E, Gehan E, Rall D, Schmidt L & Skipper H (1966) Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey and man. Cancer Chemother. Rep. **50**(4): 219-244.

Frith CH, Ward JM, Chandra M & Losco PE (2000) Non-proliferative lesions of the hematopoietic system in rats In: Guides for Toxiologic Pathology. STP/ARP/AFIP, Washington DC.

FSANZ (2009a) Principles and Practices of Dietary Exposure Assessment for Food Regulatory Purposes. http://www.foodstandards.gov.au/_srcfiles/Principles%20&%20practices%20exposure%20assessment%202009.pdf

FSANZ (2009b) Information paper: Protecting 'high consumers'. http://www.foodstandards.gov.au/scienceandeducation/scienceinfsanz/dietaryexposureassessmentsatfsanz/protectinghighconsum4441.cfm

Fulcher SM, Renaut SD & Bottomly AM (2002) ANS9801: Preliminary embryo-fetal toxicity in the rabbit by gavage administration. Second Amended Final. Report No. AJO 183/010158. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Fulcher SM, Renaut SD & Bottomly AM (2003) ANS9801: Study of effect on embryo-fetal toxicity in the rabbit by gavage administration. Report No. AJO 190/022479. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Holding JD (2004) Toxicokinetic Report. ANS9801: Toxicity study by dietary administration to beagle dogs for 52 weeks followed by a six week recovery period. Report No. AJO 196/034055. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Holding JD (2005a) Interim bioanalytical report. ANS9801: Combined carcinogenicity and toxicity study by dietary administration to Han wistar rats for 104 weeks with an *in utero* exposure phase. Report No. AJO 195/033047. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Holding JD (2005b) Toxicokinetic report. ANS9801: Combined carcinogenicity and toxicity study by dietary administration to Han wistar rats for 104 weeks with an *in utero* exposure phase. Report No. AJO 195/033048. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Horn C et al (2005) ANS9801: Combined carcinogenicity and toxicity study by dietary administration to Han Wistar rats for 104 weeks with an *in utero* exposure phase. Interim Report. Report No. AJO 195/033047. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Horne C (2006a) ANS9801. Carcinogenicity study by dietary administration to CD-1 mice for 104 weeks. Report No. AJO 198/033050. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Horne C (2006b) ANS9801: Combined carcinogenicity and toxicity study by dietary administration to Han Wistar rats for 104 weeks with an *in utero* exposure phase. Report No. AJO 195/033048. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Ito S (2007) Stability Testing of ANS9801 Bulk Substance (Long Term Testing). Report No. AM-M9-822. Lab: Institute of Applied Medicine Inc, Sapporo, Hokkaido, Japan. Sponsor: Pharmaceutical Research Laboratories, Ajinomoto Co Inc, Kawasaki, Japan. *Unpublished*.

Ito S (2008) Photostability Study of ANS9801. Report No. ANS9801-S13. Lab & Sponsor: Pharmaceutical Research Laboratories, Ajinomoto Co Inc, Kanagawa, Japan. *Unpublished*.

Jordan SM, Williams CN, Davies C & Murphy A (2001) ANS9801: Cardiovascular and respiratory evaluations in the anaesthetised dog following intraduodenal administration. Report No. AJO 163/012426. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, AminoScience Division, Tokyo, Japan. *Unpublished*.

Kato S (2004a) Determination of Solubility of ANS9801. Report No. AM-M0-454. Lab: Institute of Applied Medicine Inc, Sapporo, Hokkaido, Japan. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Kato S (2004b) Determination of Dissolution Rate of ANS9801. Report No. AM-M0-459. Lab: Institute of Applied Medicine Inc, Sapporo, Hokkaido, Japan. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Kato S (2004c) Determination of Partition Coefficient of ANS9801. Report No. AM-M0-456. Lab: Institute of Applied Medicine Inc, Sapporo, Hokkaido, Japan. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.
Kato S (2004d) Determination of Refractive Index of ANS9801. Report No. AM-M0-455. Lab: Institute of Applied Medicine Inc, Sapporo, Hokkaido, Japan. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Kato S (2004e) Determination of Specific Gravity of ANS9801. Report No. AM-M0-458. Lab: Institute of Applied Medicine Inc, Sapporo, Hokkaido, Japan. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Kato S (2004f) Determination of Viscosity of ANS9801. Report No. AM-M0-460. Lab: Institute of Applied Medicine Inc, Sapporo, Hokkaido, Japan. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Kato S (2004g) Batch Analysis of ANS9801 and ANS9801 Reference Standard. Report No. AM-M0-271. Lab: Institute of Applied Medicine Inc, Sapporo, Hokkaido, Japan. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Kato S (2004h) Batch Analysis of Arsenic Limit Test for Principal Raw Material of ANS9801 (HMCA). Report No. AM-M0-340. Lab: Institute of Applied Medicine Inc, Sapporo, Hokkaido, Japan. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Kato S (2004i) Specification Tests of ANS9801. Report No. AM-M0-200. Lab: Institute of Applied Medicine Inc, Sapporo, Hokkaido, Japan. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Kato S (2004j) Specification Tests of ANS9801 (2). Report No. AM-M0-240. Lab: Institute of Applied Medicine Inc, Sapporo, Hokkaido, Japan. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Kato S (2004k) Specification Tests of ANS9801 (3). Report No. AM-M0-270. Lab: Institute of Applied Medicine Inc, Sapporo, Hokkaido, Japan. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Kato S (2004l) Batch Analysis of ANS9801 and ANS9801 Reference Standard. Report No. AM-M0-271. Lab: Institute of Applied Medicine Inc, Sapporo, Hokkaido, Japan. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Kato S (2004m) Stability Testing of ANS9801 Bulk Substance (accelerated testing). Report No. AM-M9-821. Lab: Institute of Applied Medicine Inc, Sapporo, Hokkaido, Japan. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Kawahara S, Mori K & Amino Y (2007) Concise synthesis of ANS9801 and ANS9803; high-intensity sweeteners derived from aspartame. 2nd Conference on chemistry for life sciences, Wroclaw, Poland.

Krievins D (2005) A double-blind, randomised, placebo controlled, parallel-group study design in 24 normal healthy subjects to assess the tolerability of 4 weeks repeated administration of ANS9801. Study No. ANSE-103a. Lab: Stradins Clinical University Hospital, Riga, Latvia. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Lindinger W, Taucher J, Jordan A, Hansel A & Vogel W (1997) Endogenous Production of Methanol after the Consumption of Fruit. Alcoholism: Clinical and Experimental Research **21**: 939-943.

May K, Watson D & Burton DA (2001) ANS9801. Bacterial mutation assay. Report No. AJO 154/012404. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

McLennan W & Podger A (1998) *National Nutrition Survey Users' Guide. 1995*. (ABS Catalogue number 4801.0), Commonwealth of Australia, Canberra.

Mehmood Z, Pritchard L, Clemson AD & Davison C (2001) ANS9801. Mouse micronucleus test. Report No. AJO 160/013188. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Nagahisa S (2004a) Melting Point Determination of ANS9801. Report No. ANZ9801-P25-R0. Lab & Sponsor: Pharmaceutical Research Laboratories, Ajinomoto Co Inc, Kawasaki, Kanagawa, Japan. *Unpublished*.

Nagahisa S (2004b) pH Determination of ANS9801. Report No. ANS9801-P23-R1. Lab & Sponsor: Pharmaceutical Research Laboratories, Ajinomoto Co Inc, Kawasaki, Kanagawa, Japan. *Unpublished*.

Nagahisa S (2004c) Batch Analysis for Platinum and Palladium in ANS9801 by Inductively Coupled Plasma-Atomic Emission Spectrometry. Report No. 501090372. Lab: Tama Laboratory, Food Research Laboratories, Tokyo. Sponsor: Pharmaceutical Research Laboratories, Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Nagahisa S (2004d) Batch Analysis for Microbial Limit Test of ANS9801. Report No. 501070454 Lab: Tama Laboratory, Food Research Laboratories, Tokyo. Sponsor: Pharmaceutical Research Laboratories, Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Nagahisa S (2004e) Batch Analysis of X-Ray Powder Diffraction Measurement of ANS9801. Report No. Unspecified. Lab & Sponsor: Pharmaceutical Research Laboratories, Ajinomoto Co Inc, Kawasaki, Kanagawa, Japan. *Unpublished*.

Nagahisa S (2004f) Specification Testing of ANS9801 (Lot No. 000530). Report No. Unspecified. Lab & Sponsor: Pharmaceutical Research Laboratories, Ajinomoto Co Inc, Kawasaki, Kanagawa, Japan. *Unpublished*.

Nagahisa S (2004g) Specification Testing of ANS9801 (Lot No. 000825). Report No. Unspecified. Lab & Sponsor: Pharmaceutical Research Laboratories, Ajinomoto Co Inc, Kawasaki, Kanagawa, Japan. *Unpublished*.

Nagahisa S (2004h) Preliminary Stability Testing (Accelerated Testing) of ANS9801. Report No. ANS9801-S2-R0. Lab & Sponsor: Pharmaceutical Research Laboratories, Ajinomoto Co Inc, Kawasaki, Kanagawa, Japan. *Unpublished*.

Nagahisa S (2004i) Preliminary Stability Testing (Long Term Testing) of ANS9801. Report No. ANS9801-S1-R0. Lab: Pharmaceutical Research Laboratories, Ajinomoto Co Inc, Kawasaki, Kanagawa, Japan; Sapporo Laboratories, Institute of Applied Medicine Inc, Sapporo, Hokkaido, Japan. Sponsor: Pharmaceutical Research Laboratories, Ajinomoto Co Inc, Kawasaki, Kanagawa, Japan. *Unpublished*.

Nagahisa S (2005a) Determination of Dissociation Constant (pKa) of ANS9801. Report No. ANS9801-P40-R0. Lab & Sponsor: Pharmaceutical Research Laboratories, Ajinomoto Co Inc, Kawasaki, Kanagawa, Japan. *Unpublished*.

Nagahisa S (2005b) Batch Analysis for Lead Limit Test for ANS9801. Report No. 501090371. Lab: Tama Laboratory, Food Research Laboratories, Tokyo. Sponsor: Pharmaceutical Research Laboratories, Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Otabe A, Muraoka R & Narita T (2009a) ANS9801 Supplemental Report. Human Study ANSE-101. An open, dose escalating study to determine the safety. Tolerability, and pharmacokinetic profile of ANS9801 in healthy male volunteers. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Otabe A, Muraoka R & Narita T (2009b) ANS9801 Supplemental Report. Human Study ANSE-102. An open label study to investigate the adsorption, pharmacokinetics, metabolism and excretion of a single oral dose of 14C-ANS9801 in healthy male volunteers. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished.*

Otabe A, Muraoka R & Narita T (2009c) ANS9801 Supplementary Analysis. Human Study ANSE-103a. Safety and tolerability assessment of multiple daily doses of ANS9801: Part 1. 4-week administration to normal human subjects. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished.*

Otabe A, Muraoka R & Narita T (2009d) ANS9801 Supplementary Analyses. Human Study ANSE-103b. Safety and tolerability assessment of multiple daily doses of ANS9801: Part 2. 12-week safety study of ANS9801 administered to subjects with type 2 diabetes. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Pirage V (2006) Safety and tolerability assessment of multiple daily doses of ANS9801: Part 2 – A 12-week safety study of ANS9801 administered to subjects with type 2 diabetes. Study No. ANSE-103b. Lab: Stradins Clinical University Hospital, Riga, Latvia. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Powell LAJ & Scott AM (2005) Toxicity study by dietary administration to beagle dogs for 13 weeks followed by a 4 week recovery period. Report No. AJO 179/014664. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Powell LAJ, Lelasseux M-C & Crome SJ (2005) ANS9801: Toxicity study by oral dietary administration to beagle dogs for 52 weeks followed by a 6 week recovery period. Report No. AJO 196/034055. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Ridglan Farms (date unspecified) Blood reference values for Beagle dogs. Available online at http://ridglan.com/documents/blood.pdf

Sibley PL, Traina VM & Parekh CH (1974) Normal range and variability of hematologic and blood-cheical parameters from beagle dogs and rhesus monkeys. Toxicologic Pathology **2**: 22-27.

Saizuka T (2001) Batch Analysis for Residual Solvents of ANS9801. Report No. FR/G11085. Lab: Nagoya Laboratories, Toray Research Center Inc, Aichi, Japan. Sponsor: Ajinomoto Co. Inc., Tokyo, Japan. *Unpublished*.

Sakata H (2005) Study of Sweetness Levels of ANS9801 in Tabletop Sweetener. Report No. S-0002. Lab & Sponsor: Seasoning Development & Technology Centre, Ajinomoto Co, Kawasaki, Kanagawa, Japan. *Unpublished*.

Sakata H (2007a) Study of Sweetness Levels of ANS9801 in Powdered Soft Drink Mix. Report No. S-0005. Lab & Sponsor: Seasoning Development & Technology Centre, Ajinomoto Co, Kawasaki, Kanagawa, Japan. *Unpublished*.

Sakata H (2007b) Stability and Functionality Study of ANS9801 in Powdered Soft Drink Mixes. Report No. 1445535, 1445535, 1445536, F-0007, F-0008 & F-0009. Lab: Seasoning Development & Technology Centre, Ajinomoto Co, Kawasaki, Kanagawa, Japan; Osaka Laboratory, Sumika Chemical Analysis Service Ltd, Osaka, Japan; Stability Analysis Group, Pharmaceutical Analysis Laboratory, Sumika Chemical Analysis Service Ltd, Oita, Japan. Sponsor: Seasoning Development & Technology Centre, Ajinomoto Co, Kawasaki, Kanagawa, Japan. *Unpublished*.

Sakata H (2008) Stability and Functionality of ANS9801 in Tabletop Powder. Report No. 1437009 & F-0003. Lab: Seasoning Development & Technology Centre, Ajinomoto Co, Kawasaki, Kanagawa, Japan; Osaka Laboratory, Sumika Chemical Analysis Service Ltd, Osaka, Japan; Stability Analysis Group, Pharmaceutical Analysis Laboratory, Sumika Chemical Analysis Service Ltd, Oita, Japan. Sponsor: Seasoning Development & Technology Centre, Ajinomoto Co, Kawasaki, Kanagawa, Japan. *Unpublished*.

Tennekes H, Gembardt C, Dammann M & van Ravenzwaay B (2004) The stability of historical control data for common neoplasms in laboratory rats: adrenal gland (medulla), mammary gland, liver, endocrine pancreas, and pituitary gland. Regulatory Toxicology & Pharmacology **40**:18-27.

Warrington S (2004) An open, dose escalating study to determine the safety, tolerability and pharmacokinetic profile of ANS9801 in health male volunteers. Study No. ANSE-101. Lab: Hammersmith Medicines Research Ltd, Central Middlesex Hospital, London, UK. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Warrington S (2005) An open label study to investigate the adsorption, pharmacokinetics, metabolism and excretion of a single oral dose of ^{14}C-ANS9801 in healthy male volunteers. Study No. ANSE-102. Lab: Hammersmith Medicines Research Ltd, Central Middlesex Hospital, London, UK. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished.*

Whiteley LO, Anver MR, Botts S & Jokinen MP (2000) Proliferative lesions of the intestine, salivery glands, oral cavity, and esophagus in rats In: Guides for Toxiologic Pathology. STP/ARP/AFIP, Washington DC.

WHO (1997). Environmental Health Criteria196: Methanol. World Health Organization, Geneva.

WHO (2004) Neotame. WHO Food Additive Series 52. Available online at: http://www.inchem.org/documents/jecfa/jecmono/v52je08.htm

Williams CN & Murphy AM (2001) ANS9801: Irwin dose-range in rats following oral administration. Report No. AJO 161/012397. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, AminoScience Division, Tokyo, Japan. Unpublished.

Williams CN & Adams AR (2001) ANS9801: Assessment of locomotor activity in rats following oral administration. Report No. AJO 162/012597. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, AminoScience Division, Tokyo, Japan. Unpublished.

Williams CN, Murphy AM & Smith M (2001) ANS9801: Charcoal propulsion test in rats following oral administration. Report No. AJO 164/012575. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Sweetener Dept, Tokyo, Japan. Unpublished.

Willis S (2005a) Study of Sweetness Levels of ANS9801 in Iced Tea. Report No. 24705. Lab: Tragon Corporation, Redwood City, CA, USA. Sponsor: Seasoning Development & Technology Centre, Ajinomoto Co, Kawasaki, Kanagawa, Japan. *Unpublished*.

Willis S (2005b) Study of Sweetness of ANS9801 in Coffee. Report No. 31805. Lab: Tragon Corporation, Redwood City, CA, USA. Sponsor: Seasoning Development & Technology Centre, Ajinomoto Co, Kawasaki, Kanagawa, Japan. *Unpublished*.

Willis S (2005c) Study of Sweetness Potency of ANS9801 Compared to Aspartame in Water. Report No. 13904. Lab: Tragon Corporation, Redwood City, CA, USA. Sponsor: Seasoning Development & Technology Centre, Ajinomoto Co, Kawasaki, Kanagawa, Japan. *Unpublished*.

Willis S (2005d) Descriptive Analysis of ANS9801 Compared to Aspartame in Water. Report No. 20505. Lab: Tragon Corporation, Redwood City, CA, USA. Sponsor: Seasoning Development & Technology Centre, Ajinomoto Co, Kawasaki, Kanagawa, Japan. *Unpublished*.

Willis S (2005e) Determination of Group Difference Threshold Level (GDTL) for ANS9801 and Evaluation of ANS9801 as a Flavour Enhancer. Report No. 31905 & 35405. Lab: Tragon Corporation, Redwood City, CA, USA. Sponsor: Seasoning Development & Technology Centre, Ajinomoto Co, Kawasaki, Kanagawa, Japan. *Unpublished*.

Willoughby CR (2002) ANS9801. Study of effects on embryo-fetal development in CD rats treated by dietary administration. Report No. AJO 182/014156. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Willoughby CR (2004) ANS9801. Study of reproductive performance in CD rats treated continuously through two successive generations by dietary administration. Report No. AJO 203/033888. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. *Unpublished*.

Wing MG (2004) ANS9801. Immunology Report. Toxicity study by dietary administration to Han Wistar rats for 13 weeks followed by a 4 week recovery period. Report No. AJO 176/014075. Lab: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England. Sponsor: Ajinomoto Co Inc, Tokyo, Japan. GLP: UK, EC, OECD, US FDA & Japan. QA statement: Yes.

Appendices

Appendix 1: Standard clinical chemistry, haematology & urinalysis parameters

Clinical chemistry	Haematology	Urinalysis
albumin	blood smear	appearance
alkaline phosphatase	clotting parameters (clotting	bilirubin
bilirubin (total)	time, partial thrombin time,	chloride
calcium	activated partial thromboplastin	glucose
chloride	time)	ketones
cholesterol (total)	erythrocyte count	occult blood
creatinine (blood)	haematocrit (packed cell	pH
CPK (creatinine phosphokinase)	volume)	potassium
gamma-glutamyl transpeptidase	haemoglobin	protein
globulin	leucocyte differential count	reducing substances
glucose (blood)	leucocyte total count	specific gravity
LDH (serum lactate dehydrogenase)	MCH (mean corpuscular	sediment
OCT (ornithine carbamyl transferase)	haemoglobin)	(microscopic)
phosphorus	MCHC (mean corpuscular	sodium
potassium	haemoglobin concentration)	urobilinogen
protein (total)	MCV (mean cell volume)	volume
SGPT (serum alanine	platelet count	
aminotransferase)	reticulocyte count	
SGOT (serum aspartate		
aminotransferase)		
sodium		
triglycerides		
urea nitrogen (blood)		

Appendix 2: Standard organs/tissues for weight determination & histopathology

Organ weights	Histopathology		
Adrenals Brain Gonads Heart Kidneys Liver Spleen Thymus Thyroid (w/parathyroid)	Adrenals aorta blood smear bone bone marrow brain (3 levels) cecum colon duodenum epididymes eyes eyes (optic nerve) gall bladder Harderian/nictitans glands head - 3 sections (nasal cavity, paranasal sinus, tongue, oral cavity, nasopharynx, inner-ear)	heart ileum jejunum kidneys lachrymal gland liver lungs lymph nodes mammary gland muscle (smooth) muscle (skeletal) nerve (peripheral) oesophagus ovaries pancreas pituitary	prostate rectum salivary gland seminal vesicle skin spinal cord (cervical thoracic, lumbar) spleen sternum stomach testes thymus thyroid (w/parathyroid) trachea urinary bladder uterus vagina Zymbal's gland (rodents) gross lesions

Appendix 3: Standard reproduction indices

MATING INDEX

$$\frac{\text{No. animals mating}}{\text{Animals paired}} \times 100$$

CONCEPTION RATE

$$\frac{\text{No. pregnant females}}{\text{Animals mated}} \times 100$$

FERTILITY INDEX

$$\frac{\text{No. pregnant females}}{\text{Animals paired}} \times 100$$

POST IMPLANTATION SURVIVAL INDEX

$$\frac{\text{Total No. offspring born}}{\text{Total No. uterine implantation sites}} \times 100$$

LIVE BIRTH INDEX

$$\frac{\text{No. live offspring on Day 1}}{\text{Total No. offspring born}} \times 100$$

VIABILITY INDEX

$$\frac{\text{No. live offspring on Day 4 (before culling)}}{\text{No. live offspring on Day 1}} \times 100$$

LACTATION INDEX

$$\frac{\text{No. live offspring on Day 21}}{\text{No. live offspring on Day 4 (after culling)}} \times 100$$

Appendix 4: Dietary Exposure Assessments at FSANZ

A dietary exposure assessment is the process of estimating how much of a food chemical a population, or population sub group, consumes. Dietary exposure to (or intake of) food chemicals is estimated by combining food consumption data with food chemical concentration data. The process of doing this is called 'dietary modelling'.

Dietary exposure = food chemical concentration x food consumption

FSANZ's approach to dietary modelling is based on internationally accepted procedures for estimating dietary exposure to food chemicals. Different dietary modelling approaches may be used depending on the assessment, the type of food chemical, the data available and the risk assessment questions to be answered. In the majority of assessments FSANZ uses the food consumption data from each person in the national nutrition surveys to estimate their individual dietary exposure. Population summary statistics such as the mean exposure or a high percentile exposure are derived from each individual person's exposure.

An overview of how dietary exposure assessments are conducted and their place in the FSANZ Risk Analysis Process is provided on the FSANZ website at:

http://www.foodstandards.gov.au/scienceandeducation/scienceinfsanz/dietaryexposureassessmentsatfsanz/dietaryexposureandin4438.cfm

FSANZ has developed a custom built computer program 'DIAMOND' to calculate dietary exposures. More information on DIAMOND is available on the FSANZ website at:

http://www.foodstandards.gov.au/scienceandeducation/scienceinfsanz/dietaryexposureassessmentsatfsanz/fsanzdietaryexposure4439.cfm

Further detailed information on the principles and practices of conducting dietary exposure assessments at FSANZ is provided in *Principles and Practices of Dietary Exposure Assessment for Food Regulatory Purposes* (FSANZ, 2009), available at:

http://www.foodstandards.gov.au/_srcfiles/Principles%20&%20practices%20exposure%20assessment%202009.pdf

A4.1 Food consumption data used

The most recent food consumption data available were used to estimate exposures to Advantame for the Australian and New Zealand populations. The national nutrition survey (NNS) data used for these assessments were:

- The 2007 Australian National Children's Nutrition and Physical Activity Survey (also known as '*Kids Eat Kids Play*') (2007 AusNNS)
- The 1995 Australian National Nutrition Survey (1995 AusNNS).
- The 1997 New Zealand National Nutrition Survey (1997 NZNNS)

The results for Australian children aged 2-16 years were reported using the 2007 AusNNS and for the population 17 years and above used the 1995 AusNNS. The design of each of these surveys varies somewhat and key attributes of each are set out below.

A4.1.1 2007 Australian Children's Nutrition & Physical Activity Survey (2007 AusNNS)

The 2007 AusNNS collected data on nutrition and physical activity for 4,487 children aged 2-16 years across Australia. The survey was conducted over a seven month time period, from February to August 2007.

In contrast to other national nutrition surveys used to date by FSANZ (the 1995 Australian and 1997 New Zealand surveys), in the 2007 AusNNS each respondent completed two 24-hour recalls on non-consecutive days. The availability of two days of food consumption data provides a more realistic estimate of long term consumption of infrequently consumed foods, because it takes account of those who may eat a food on one day of the survey but not on the other. Using one 24-hour recall may capture an unusual eating occasion for an individual that does not describe how they normally eat.

In this assessment, exposure to Advantame was estimated from each consumer's average exposures from foods containing Advantame across Day 1 and Day 2. The results of the 2007 AusNNS were weighted to represent the overall population of Australian children because stratified sampling with non-proportional samples was used.

A4.1.2 1995 Australian National Nutrition Survey (1995 AusNNS)

The 1995 AusNNS provides comprehensive information on dietary patterns of a sample of 13,858 Australians aged from 2 years and above (McLennan & Podger 1998). It is the most recent NNS for Australians aged 17 years and above. The survey used a 24-hour recall method for all respondents, with 10% of respondents also completing a second 24-hour recall on a second, non-consecutive day. Food frequency data are available for a subset of the national sample (respondents aged 12 years and above) as are responses to a series of short dietary questions about food habits. These data are used unweighted in DIAMOND.

A4.1.3 1997 New Zealand National Nutrition Survey (1997 NZNNS)

The 1997 NZNNS provides comprehensive information on the dietary patterns of a sample of 4,636 respondents aged from 15 years and above. The survey was conducted on a stratified sample over a 12 month period. The survey used a 24-hour recall methodology with 15% of respondents also completing a second 24-hour recall with an additional food frequency questionnaire and questions on food consumption patterns. These data are used unweighted in DIAMOND.

The 2002 New Zealand Children's National Nutrition Survey is not yet available through DIAMOND and therefore could not be used for this dietary exposure assessment.

Further information on the National Nutrition Surveys used to conduct dietary exposure assessments is available on the FSANZ website at:

http://www.foodstandards.gov.au/scienceandeducation/scienceinfsanz/dietaryexposureassessmentsatfsanz/foodconsumptiondatau4440.cfm

A4.2 Change in approach for 'high consumers'

Because of the exaggeration of extremes of consumption that arise where estimates of dietary exposure are based on food consumption data from one or two days of single 24-hour recall from NNSs, FSANZ has adopted a policy that a high consumer's chronic dietary exposure is best represented by the 90th percentile of exposure. This replaces the previous standard use of the 95th percentile and is in line with international best practice. For further information on the use of the 90th percentile for dietary exposure assessments, refer to the

FSANZ information paper: Protecting ‘high consumers’ (FSANZ 2009b).

For more information on FSANZ dietary exposure assessment principles, methodology, assumptions and limitations and uncertainties of the concentration and food consumption data, see the FSANZ document, Principles and Practices of Dietary Exposure Assessment for Food Regulatory Purposes (FSANZ 2009a).

A4.3 Limitations of dietary exposure assessments

Dietary exposure assessments based on 2007 AusNNS, 1995 AusNNS and 1997 NZNNS food consumption data provides the best estimate of actual consumption of a food and the resulting estimated dietary exposure assessment for Australian children 2-16 years, Australian adults 17 years and above, and the New Zealand population aged 15 years and above, respectively. However, it should be noted that NNS data do have limitations. Further details of the limitations relating to dietary exposure assessments undertaken by FSANZ are set out in the FSANZ document, *Principles and Practices of Dietary Exposure Assessment for Food Regulatory Purposes* (FSANZ 2009a).

Appendix 5: Dietary Exposure Assessments

TABLE A5.1: ESTIMATED DIETARY EXPOSURES TO ADVANTAME

Survey	Age group	Number of consumers	Consumers as a % of Respondents	Dietary Exposure (mg/day)	
				Mean	P90
2007 Aus NNS	2-6 years	1312	90	1.2	2.6
	7-16 years	2774	92	1.5	3.3
1995 Aus NNS	17 years and over	10101	91	2.7	5.0
1997 NZNNS	15 years and over	4237	91	2.2	4.2

TABLE A5.2: ESTIMATED DIETARY EXPOSURES TO ADVANTAME AS A PERCENTAGE OF THE ADI

		mg/kg bw/day		%ADI (ADI = 5 mg/kg bw/day)	
Survey	Age group	Mean	P90	Mean	P90
2007 Aus NNS	2-6 years	0.06	0.14	1.3	2.8
	7-16 years	0.04	0.08	0.7	1.5
1995 Aus NNS	17 years and over	0.04	0.07	0.7	1.4
1997 NZNNS	15 years and over	0.03	0.06	0.6	1.2

TABLE A5.3: MAJOR CONTRIBUTORS TO ESTIMATED ADVANTAME EXPOSURE

	% contribution to advantame exposure			
	Australia			New Zealand
Food group	2-6 years[1]	7-16 years[1]	17 years & over[2]	15 years & over[3]
Liquid milk products & flavoured liquid milk	60	45	4	1
Tabletop sweeteners	<1	<1	1	1
Tea	1	5	48	63
Beverages, made up from flavouring	8	13	<1	<1
Formulated meals & sports foods	12	17	<1	<1
Cordials, electrolyte and sports drinks	19	19	10	12
Coffee beverages	<1	<1	35	22

NC = Not consumed
1. 2007 Aus NNS
2. 1995 Aus NNS
3. 1997 NZNNS