ANS9801

PRELIMINARY EMBRYO-FETAL TOXICITY STUDY

IN THE RABBIT BY ORAL GAVAGE ADMINISTRATION

SECOND AMENDED FINAL REPORT

Sponsor

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Final: 5 February 2002
First Amended Final: 16 April 2002
Second Amended Final: 11 May 2005

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AUTHOR'S STATEMENT

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I, the undersigned, hereby declare that the work presented in this report was performed under my supervision according to the procedures herein described and that this report provides a correct and faithful record of the results obtained.

The study described in this report generally followed the Good Laboratory Practice principles but study design and the report do not contain all the elements required by GLP. No specific study-related Quality Assurance procedures were performed and no analysis of dosage form was conducted.

I consider the data generated to be valid for the intended purpose of selection of dose levels for further studies.

The first amended final report was issued to incorporate changes agreed between the Study Director and the Sponsor on pages 61, 80, 81 and 83 that were not printed correctly in the original final report. Subsequent to the issue of that report it became apparent that the values quoted for ANS 9801 and ANS 9801-acid were incorrect, as no allowance had been made for the citric acid added to the plasma.

At the request of the Sponsor, this second amended report was issued to include the corrections required to the toxicokinetic data with respect to possible re-esterification of the acid metabolite and deconjugation of acid sulphate conjugate under conditions of extraction for plasma concentration analysis. All concentrations originally obtained for ANS9801 and ANS9801-acid were multiplied by a factor of 1.1 to account for the citric acid added to the plasma. The statistical package used to calculate the AUC24 was also upgraded from version 6.12 to 8.2.

The partial validation in rabbit plasma was ongoing at the time of analysis of the samples from AJO/183, therefore not all stability experiments had been performed. When the Day 6 samples were analysed they had been kept for longer than 1 week, which, at the time, was the actual verified stability period. However, at the time of reporting the data from this study, ANS9801 and ANS9801-acid were shown to be stable for 3 months, which covers the storage period of all samples analysed, and this is considered not to have affected the integrity of the study.

S.M. Fulcher

11 May 2005

S.M. Fulcher, B.A., F.I.A.T.
Study Director
Huntingdon Life Sciences Ltd.

Date

CONTRIBUTING SCIENTISTS

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STUDY MANAGEMENT

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SUMMARY

Procedures

The objective of this preliminary study was to assess the effects of ANS9801, a food sweetener, upon the progress and outcome of pregnancy in rabbits of the New Zealand White strain. The results of this study are intended to establish suitable dosages for a main embryo-fetal toxicity study.

For this purpose, ANS9801 was administered once daily by the oral (gavage) route at dosages of 500, 1000 and 2000 mg/kg/day to four groups of six animals from Day 6 to Day 28 after mating inclusive. Control animals received the vehicle, 1% methylcellulose, over the same treatment period.

Blood samples were taken for toxicokinetic analysis from all animals, on Days 6 and, where possible, 27 after mating in order to establish the toxicokinetic profile for ANS9801. Samples were analysed by the Department of Mass Spectrometry, Huntingdon Research Centre using a method determined in a separate study in this package of work (Huntingdon Life Sciences Report Number AJO/170/014335).

On Day 29 after mating all surviving animals were subjected to a detailed necropsy procedure for examination of their uterine contents and fetuses were examined externally for evidence of any abnormalities.

Results

At 2000 mg/kg/day, one female was killed for humane reasons on Day 21 of gestation after showing marked reddening and swelling of the ano-genital region which was confirmed as pronounced and reddened labia at necropsy. In isolation this finding was not conclusively related to treatment.

In addition to this death, one Control female and one female at 500 mg/kg/day showed evidence of abortion and were killed on Day 20 of gestation. Both animals had a single implantation only, which was considered a likely contributory factor.

Almost all animals in groups treated with ANS 9801 showed green/purple/pink staining of the cage tray paper at some point during treatment. There were also sporadic incidences of green/pink/purple urine being observed for treated animals. This colouration of the urine is thought to be due to the test material or a metabolite, but the specific aetiology is unknown.

There were no post-dose observations observed in any animal throughout the treatment period

There was no marked effect of treatment with ANS 9801 upon group mean values of bodyweight, bodyweight change, food or water consumption. It was noted however that females at 2000 mg/kg/day were slower to return to a pattern of bodyweight gain following the slight non-specific weight loss that occurred in all groups after the start of treatment. There was inter-group variation in gravid uterine weight and adjusted bodyweight/bodyweight change but, no clear effect of treatment was observed.

Macroscopic necropsy examination of females on Day 29 after mating revealed no consistent findings that were considered to be related to treatment with ANS 9801.

No clear effect of treatment was observed on litter parameters, as assessed by the number of implantations, resorptions, live young, sex ratio and pre- and post-implantation loss, or on placental, litter or fetal weights at any of the dosages investigated. The inter-group variation in litter weights observed was considered to be due to occasional animals having very small litters.

There were no consistent macroscopic findings among fetuses that would suggest a clear adverse effect of treatment upon morphological development.

The toxicokinetics revealed that animals in all groups receiving ANS 9801 were exposed to ANS 9801 and ANS 9801 - acid.

CONCLUSION

In this study, dosages of up to 2000 mg/kg/day were well tolerated in the pregnant rabbit, and the minimal signs of toxicity observed did not preclude the high dosage of 2000 mg/kg/day from use in the main embryo-fetal study. 2000 mg/kg/day is also considered to be the maximum practical dosage in the rabbit with this material under the conditions of use in this study. It is concluded that the dosage sequence applied in this preliminary study, 0 (Control), 500, 1000 and 2000 mg/kg/day would also be suitable for use in the main embryo-fetal development study.

INTRODUCTION

Objective

The objective of this preliminary study was to assess the effects of ANS9801, a food sweetener, upon the progress and outcome of pregnancy in the rabbit. ANS9801 was administered daily to the dam during the organogenesis phase of gestation and continued to shortly before the expected day of delivery to cover the fetal phase of gestation. Plasma samples were obtained from the dams on the first day of treatment (Day 6) and Day 27 of gestation in order to establish the toxicokinetic profile for ANS9801. The data generated in this study are intended to establish suitable dosages for use in a main embryo-fetal study.

Regulatory compliance

The study was performed taking into account the U.S. Food and Drug Administration, Centre for Food Safety and Applied Nutrition Redbook 2000. Toxicological Principles for the Safety of Food Ingredients, Guidelines for Developmental Toxicity Studies, (Section IV.C.9.b) recommend exposure from at least the day of implantation to the day prior to the day of expected parturition; as this will be the intended regime for the main study, the same treatment period was adopted for this study.

The work performed in this study generally followed good laboratory practice principles, however, no specific study-related Quality Assurance procedures were performed, and this report may not contain all of the elements required by GLP.

Justification for the test system

The rabbit was chosen because it satisfies the requirements of the regulatory agencies for embryo-fetal development studies. The New Zealand White rabbit was used because of the historical control data available in this laboratory.

Justification for the treatment regimen

The oral (gavage) route was selected as this is the primary route of human exposure.

Dosages of 0 (Control), 500, 1000 and 2000 mg/kg/day were specified by the Sponsor on the basis of a tolerance study (Huntingdon Life Sciences Report No. AJO166/010068) in which 2000 mg/kg/day was well tolerated for 14 days consecutive dosing. A dosage level of 2000 mg/kg/day is also considered to be the maximum practical dosage for the rabbit for this test material.

Study organisation

Testing facilities:

The principal laboratory was:

Huntingdon Life Sciences
Eye Research Centre
Eye
Suffolk
IP23 7PX
ENGLAND

Bioanalysis was performed by:

Department of Mass Spectrometry
Huntingdon Research Centre
Huntingdon
Cambridgeshire
PE28 4HS
England.

Study timing:

Experimental start date (Protocol Issue)	:	04 July 2001
Animals arrived	:	12 July 2001
Treatment commenced	:	13 August 2001
Experimental completion date (final necropsy)	:	05 September 2001

MATERIALS AND METHODS

Design conditions

Animals

A total of twenty six sexually mature virgin female New Zealand White rabbits, approximately 15-17 weeks old, were obtained from an accredited closed colony, Highgate Farm, Market Rasen, Lincolnshire, England, for use in this investigation.

The animals were allowed an initial twelve days acclimatisation at Huntingdon Life Sciences during which time they were examined daily to check their physical condition and weighed at least once each week. However, assessment of females prior to pairing indicated that many of the animals were not sharing signs consistent with receptiveness to mating, so acclimatisation was extended to a total of 26 days. Following acclimatisation, animals were naturally mated on a 1:1 basis with New Zealand White stock males of established fertility; once a male had successfully mated it was not used for further mating within this study. All females were mated on a single day and following mating, each female was injected intravenously with 25 i.u. of luteinising hormone to promote ovulation.

Females were uniquely identified before arrival by the animal supplier using indelible numbers written in the ear. This identification number was used until the time of allocation to the study when each animal was given a study number ear tag.

A total of 24 animals were allocated to the study. Animals were allocated on the day of mating (Day 0 of gestation) in such a way as to avoid, as far as possible, siblings being allocated to the same treatment group. At allocation to the study, the twenty four animals used were in the bodyweight range of 3.59 – 4.62 kg and were approximately 18-22 weeks old.

The two spare animals were not allocated to the study and were not dosed. After mating, the males were returned to the stock colony and are not considered to be part of the study.

Environmental control

The animals were housed in a limited access rabbit facility.

The animal room had its own supply of filtered air, which was not recirculated, providing a minimum of 12 air changes per hour. The temperature in the animal room was controlled and values were recorded daily; the target range was 15-23°C. Relative humidity (RH) was monitored and recorded daily; the expected range was 40-70% RH. Achieved ranges of temperature and relative humidity were 17-24.5°C and 50-72% RH respectively. Copies of these data have been filed in the archives of Huntingdon Life Sciences, Eye, Suffolk. On 4 occasions temperature was slightly higher than the target range, each time not exceeding 24.5° C. These excursions all occurred prior to treatment and are considered to have had no effect on the integrity of the study. Relative humidity target ranges were exceeded on two occasions, with 71.5 % RH being the maximum value obtained. These minor deviations were again considered not to have affected the integrity of the study.

Lighting was controlled to provide a 14-hour light: 10-hour dark cycle operated with the lights on at 06.00 GMT.

Alarms were set to activate if there was any failure of the ventilation system or temperature limits were exceeded. A stand-by electricity supply was available to be automatically brought into operation should the public supply fail.

Animal accommodation

Rabbits were housed singly in suspended plastic cages (Labcare Precision Cages, Aldington Kent, England) mounted in batteries. The cages were fitted with perforated counter-sunk floor panels. An undertray beneath the floor was lined with absorbent paper, which was changed at least three times per week.

Diet and water supply

A commercially available laboratory animal diet, S.Q.C. Standard Rabbit Diet (Special Diets Services Limited, Witham, Essex, England) was freely available throughout the study. The manufacturer supplied a Certificate of Analysis with every batch, which is filed in the archives of Huntingdon Life Sciences, Eye, Suffolk.

Tap water from the domestic supply was supplied to the cages via polyethylene water bottles with sipper tubes. In England the supply and quality of this water are governed by the Department of the Environment regulations. Certificates of analysis are routinely received from the supplier (Essex and Suffolk Water plc, Chelmsford, Essex, England) and have been retained in the archives of Huntingdon Life Sciences, Eye, Suffolk.

No contaminants were reasonably expected to be present in either the diet or the water at levels known to be capable of interfering with the purpose and outcome of this study.

TREATMENT

Test article

A consignment of 35 Kg of ANS9801 (N-[N-[3-(3-hydroxy-4-methoxyphenyl)propyl]-α-aspartyl]-L-phenylalanine 1-methyl ester, Batch 000825) was received at Huntingdon Life Sciences, (Huntingdon Research Centre) on 11 December 2000 of which 1000 g was transferred to Eye Research Centre on 24 January 2001. A total of approximately 2770 g of the test article was used on this study.

Before test article use the identity, strength, purity and composition or other characteristics which appropriately defined the batch from which the test article for this study was drawn, were determined by the Sponsor. Stability of the test article and methods of synthesis, fabrication or derivation were documented by the Sponsor. A certificate of analysis is presented in Addendum 1.

Before the test article was used a 1g representative sample was taken from this consignment. This samples were each placed in a well-closed container and stored in the archives under the conditions specified for the bulk of the test article.

All dosages and concentrations are expressed in terms of the test material received.

Formulation method

Formulations were prepared in 1% Methylcellulose using the following methodology. For each concentration an appropriate amount of ANS9801 was ground using a pestle and mortar. An appropriate amount of the vehicle was placed on top of the test article, which was then ground again, ensuring a thorough mixing of the test article and vehicle. The mixture was then transferred to an appropriate measuring cylinder (rinsing the mortar with vehicle) and made to volume with the required amount of vehicle. The formulation was then transferred to a beaker/jar and mixed with a magnetic stirrer for at least five minutes.

ANS9801 had been shown to be stable in 1% Methylcellulose formulations for at least 15 days at refrigeration temperature (approximately 4°C) when protected from the light (Huntingdon Life Sciences Report No. AJO149/004663); therefore, formulations used in this study were prepared in batches of up to seven days and aliquoted for daily use. Formulations were stored in glass amber bottles at approximately 4°C and protected from light until use.

Group	Treatment	Dosage# (mg/kg/day)	Concentration@ (mg/ml)	Dose volume (ml/kg)
1	Control	0	0	5
2	ANS9801	500	100	5
3	ANS9801	1000	200	5
4	ANS9801	2000	400	5

@ Material as supplied.

Composition of treatment groups and dose administration

Animals were assigned to the groups as follows:

Group	Treatment	Dosage (mg/kg/day)	Number of females	Animal numbers
1	Control	0	6	1-6
2	ANS9801	500	6	7-12
3	ANS9801	1000	6	13-18
4	ANS9801	2000	6	19-24

Animals received the test material or vehicle Control formulations once daily, by oral gavage administration (gastric intubation), from Day 6 to Day 28 after mating at a dosage volume of 5 ml/kg bodyweight/day. The volume administered to each animal was calculated from the bodyweight recorded immediately before each administration. As far as possible animals were dosed at approximately the same time each day.

Formulations were removed from refrigeration, allowed to attain ambient temperature and stirred using a magnetic stirrer before and throughout the dosing procedure.

Control animals received the vehicle at the same volume-dosage as the treated groups during the same treatment period.

SERIAL OBSERVATIONS

Clinical signs

All animals were observed daily throughout the study and any visible signs of reaction to treatment were recorded with details of type, severity, time of onset and duration.

Detailed observations were performed during the dosing period; these observations were recorded at the following times in relation to dosing:

Pre-dose.
At the end of dosing all groups.
Approximately ½ hour after dosing.
Approximately 1 and 2 hours after dosing.
Approximately 4 hours after dosing, or as late as possible in the working day.

During the acclimatisation period, observations of the animals and their cages were recorded at least twice per week.

Mortality and abortion

One animal at 2000 mg/kg/day was killed for humane reasons on Day 21 after mating and subjected to a detailed macroscopic necropsy investigation where any abnormalities observed were retained in the appropriate fixative.

In addition, two animals (one receiving 500 mg/kg/day and one belonging to the Control group) were observed to have aborted their litter on Day 20 after mating and were subsequently killed and subjected to a detailed macroscopic necropsy examination where any abnormalities observed were retained in 10% Neutral Buffered Formalin (4% Formaldehyde).

Bodyweights

Bodyweights were recorded for each animal on a daily basis throughout the study.

Food consumption

Food consumption was recorded for each animal on a daily basis throughout the study.

Water consumption

Water consumption was recorded over a 24-hour period for each of Days 1, 8, 13, 20 and 26.

Toxicokinetics

Blood samples were obtained, from all study animals on the first day of treatment (Day 6) and, where possible, on Day 27 after mating as follows:

Group	Dosage	Time of sampling (hours after dosing)							
		Pre-dose	0.5	1	2	4	8	12	24
	(mg/kg/day)	Animal numbers							
1	Control	1-3	4-6	1-3	4-6	1-3	4-6	1-3	4-6
2	500	7-9	10-12	7-9	10-12	7-9	10-12	7-9	10-12
3	1000	13-15	16-18	13-15	16-18	13-15	16-18	13-15	16-18
4	2000	19-21	22-24	19-21	22-24	19-21	22-24	19-21	22-24

Where possible, approximately 1 ml of blood was collected into sample tubes from the central auricular artery. The anticoagulant used was Lithium heparin + 6µl diethyl-p-nitrophenyl phosphate (Paraoxan) in ethanol (5 mg/ml). Blood samples from treated animals were centrifuged at approximately 1000 x g and 4º C for a period of approximately 10 minutes. The plasma was removed, placed into appropriately

labelled plastic tubes and transferred to a freezer (at approximately -70° C), prior to despatch to the Department of Mass Spectrometry, Huntingdon Research Centre, Huntingdon, Cambridgeshire PE28 4HS, for Bioanalysis. Samples taken from Control animals were taken for procedural purposes only and were not analysed. On the second sampling occasion, blood samples were not obtained from animals 1 (Control group), 10 (500 mg/kg/day) and 24 (2000 mg/kg/day), as these animals were killed before the toxicokinetic bleeds on Day 27 after mating. Results of the toxicokinetic analyses are presented in Addendum 2.

TERMINAL STUDIES

Litter responses

On Day 29 after mating females were killed by intravenous injection of pentobarbitone sodium for examination of their uterine contents. Each animal was first examined macroscopically for evidence of disease or adverse reaction to treatment and specimens of tissues considered abnormal were retained in 10% neutral buffered formalin (4% formaldehyde). The reproductive tract, complete with ovaries, was dissected out and for pregnant animals the following recorded:

a) Gravid uterine weight;

b) Number of corpora lutea in each ovary;

c) Number of implantation sites. In apparently non-pregnant animals, the absence of implantation sites was checked using the Salewski staining technique (Salewski, E,; *Naunyn Schmied. arch. exp. Pathol*. Pharmacol., 247:367, 1964);

d) Number of resorption sites (classified as early or late);

e) Number and distribution of live and dead fetuses in each uterine horn

Fetal examination

External

All fetuses were killed by subcutaneous injection of pentobarbitone sodium and the following were recorded:

a) Weight of individual fetuses;

b) Weight of individual placentae;

c) External abnormalities of individual fetuses and placentae.

Internal

The neck and the thoracic and abdominal cavities of all fetuses from each litter were dissected, the contents examined and sex recorded. Following examination, the fetuses were eviscerated and one third of the fetuses in each litter were decapitated and their heads stored in Bouin's fluid. Torsos and the remaining intact fetuses were stored in industrial methylated spirit (74° o.p).

TREATMENT OF DATA

Data were expressed as means with standard deviations (SD), where appropriate, calculated according to the formula:

$$SD = \sqrt{\frac{\Sigma(x - \bar{x})^2}{n - 1}}$$

where x = individual or litter mean values
$\bar{x}$ = group mean value
n = sample size

unless otherwise indicated.

Clinical signs

Clinical signs that were recorded before the start of treatment were largely limited to yellow staining of the fore and hindpaws and tail. Also, many animals showed signs such as bruising or red (blood) staining of the pinnae. Clinical signs of yellow staining that began before treatment commenced, and bruising or red (blood) staining of the pinnae, which were considered to be a direct result of the toxicokinetic bleeds, were not reported in order to differentiate between clinical signs routinely observed in rabbits and signs that could be related to treatment.

Maternal bodyweight

Individual values were presented and group mean values and SD calculated for animals with live young at termination for Days 0, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26 and 28 of gestation. Weight changes were calculated, and also plotted graphically, with respect to Day 6 of gestation. Bodyweight and bodyweight change values were also presented, and group mean values and SD calculated for animals with live young at termination, for Day 29 and Days 6-29, after being adjusted according to gravid uterine weight.

Food consumption

Individual values were presented and group mean values and SD calculated for animals with live young at termination daily during gestation.

Water consumption

Individual values were presented and group mean values and SD calculated for animals with live young on Day 29 of gestation for Days 1, 8, 13, 20 and 26 after mating. At the end of the first day of water consumption measurements, water bottles were found to be empty due to the animals playing with them. Due to this, phases of water consumption for particular animals were voided, as an accurate reflection of how much water the animal would have consumed could not be gained.

Litter responses

The litter was considered the primary unit of assessment and therefore group mean values and SD were calculated from the individual litter values.

Pre-natal losses were considered separately for the pre- and post-implantation phases. Pre-implantation loss was considered to include losses due to non-fertilisation of ova and very early post-

implantation deaths (i.e. those occurring during the first two to three days post-implantation), in addition to true pre-implantation loss. It was calculated from the formula:

$$\frac{\text{(Number of corpora lutea – Number of implantations)}}{\text{Number of corpora lutea}} \times 100$$

Post-implantation loss was considered not to include the first two to three days post-implantation as any deaths that occurred in this phase would probably leave no remains visible at Day 29. It was calculated from the formula:

$$\frac{\text{(Number of implantations – Number of live fetuses)}}{\text{Number of implantations}} \times 100$$

Mean fetal weights were calculated for each sex. Overall fetal weights and placental weights, and the total litter weight for live fetuses were also calculated. Individual sex ratio was calculated on a percentage male basis for individual litters.

Fetal observations

Fetuses observed to have macroscopic findings were listed individually. Only fetuses with findings are presented.

Statistical evaluation

With the exception of the toxicokinetic analyses, the small sample size was considered to preclude meaningful statistical evaluation. Details relating to statistical analysis of toxicokinetic data are presented in the relevant section of the Toxicokinetic Report (Addendum 2).

Archives

All experimental data arising from the study (including documentary raw data, specimens, records, and other materials; collectively defined as the "materials") will remain the property of the Sponsor.

Samples and materials which are unsuitable, by reasons of instability, for long term retention and archiving may be disposed of after the periods defined in Huntingdon Life Sciences Standard Operating Procedures have been completed.

All other samples, specimens and all raw data will be retained by Huntingdon Life Sciences in its archive for a period of 5 years from the date of issue of the final report. After such time, the Sponsor will be contacted and their advice sought on the return, disposal or further retention of the materials. If requested, Huntingdon Life Sciences will continue to retain the materials subject to a reasonable fee being agreed with the Sponsor.

Huntingdon Life Sciences will retain a copy of the final report in its archives indefinitely.

RESULTS

MATERNAL RESPONSES

Mortality and abortions (Appendix 1)

At 2000 mg/kg/day, one female was killed for humane reasons on Day 21 of gestation after showing marked reddening and swelling of the ano-genital region. Macroscopic examination revealed pronounced and reddened labia and that the animal was pregnant. This animal had also shown a general decrease in bodyweight and food consumption from Day 12 after mating, along with few, loose faeces from Day 16 after mating. Given the isolated nature of this occurrence a relationship to treatment is considered unproven.

In addition to this death, one Control group female and one female at 500 mg/kg/day both showed evidence of abortion and were subsequently killed on Day 20 of gestation. Macroscopic examination revealed a single implantation in the uterus of each animal. Abortion is not uncommon in animals with a very low litter size, and no association with treatment was indicated

Clinical signs (Appendix 2)

Almost all animals in groups treated with ANS 9801 showed green/purple/pink staining of the cage tray paper at some point during treatment. There were also sporadic incidences of green/pink/purple urine being observed for treated animals. This colouration of the urine, and consequent staining of the cage tray paper, is thought to be due to the test material or a metabolite, but the specific reaction involved is unknown.

There were no other consistent clinical observations that were considered to be related to treatment.

There were no post-dose observations observed in any animal throughout the treatment period.

Maternal bodyweight (Figure 1; Tables 2 and 3; Appendix 3)

There was no marked effect of treatment with ANS 9801 upon group mean values of bodyweight or bodyweight change. It was noted however that females at 2000 mg/kg/day were slower to return to a pattern of weight gain following the slight non-specific, weight loss that occurred in all groups after the start of treatment.

Gravid uterine weight and adjusted bodyweight/bodyweight change (Table 4; Appendix 4)

There was noticeable inter group variation for gravid uterine weight and adjusted bodyweight/bodyweight change, however, no clear effect of treatment was observed.

The lower gravid uterus weight for animals at 500 mg/kg/day was principally due to a lower litter size, and consequently lower litter weight, however, adjusted bodyweight and adjusted bodyweight change were unaffected. Conversely females receiving 1000 mg/kg/day showed a higher gravid uterine weight due to a greater litter size, and consequently litter weight, (compared to Controls), with a slight reduction in adjusted bodyweight and adjusted bodyweight change values. As group mean values of gravid uterine weight, adjusted bodyweight and adjusted bodyweight gain for females at 2000 mg/kg/day were essentially similar to Controls, these lower adjusted bodyweight values at 1000 mg/kg/day were not considered to be related to treatment.

Food consumption (Table 5; Appendix 5)

There was no obvious or consistent effect of treatment with ANS 9801 upon group mean values for food consumption.

Water Consumption (Table 6; Appendix 6)

As was to be expected there was much inter-group variation for group mean values of water consumption, however, no clear adverse effect of treatment with ANS 9801 was apparent.

Necropsy findings (Appendix 7)

Macroscopic necropsy examination of females on Day 29 after mating revealed no consistent findings that were considered to be related to treatment with ANS 9801.

The green coloration of the contents of the urinary bladder observed for some animals was considered to be related to excretion of the test material or a metabolite rather than any adverse effect of treatment.

LITTER RESPONSES

In addition to the mortality and the two abortions previously mentioned, two animals were found not to be pregnant at termination; one in the Control group and one at 2000 mg/kg/day. The following assessment is based on 4, 5, 6 and 4 animals at 0 (Control), 500, 1000 and 2000 mg/kg/day respectively with live young at Day 29 of gestation.

Litter data (Table 7; Appendix 8)

There was great inter-litter variation within the study, however, no clear adverse effect of treatment on litter parameters was observed, as assessed by the number of implantations, resorptions, live young, sex ratio and pre- and post-implantation loss at any of the dosages investigated.

At 500 and 2000 mg/kg/day, pre-implantation loss was higher than the Control, however, as treatment is considered to have commenced after implantation, a relationship to treatment is considered unlikely.

At 2000 mg/kg/day, post-implantation loss was also slightly higher than the Control. Following a comparison of individual litter values however, a treatment-related effect was considered to be unproven.

Placental, litter and fetal weight (Table 8; Appendix 9)

There was no clear adverse effect of treatment with ANS 9801 upon placental, litter or fetal weights. Inter-group variation in litter weights was considered to be due to occasional animals having very small litters.

Fetal evaluation (Appendix 10)

There were no consistent macroscopic necropsy findings observed that would suggest a clear adverse effect of treatment upon the fetuses.

TOXICOKINETICS (Addendum 2)

The toxicokinetics revealed that animals in all groups receiving ANS 9801 were exposed to ANS 9018 and ANS 9801 - acid.

CONCLUSION

In this study, dosages of up to 2000 mg/kg/day were well tolerated in the pregnant rabbit, and the minimal signs of toxicity observed did not preclude the high dosage of 2000 mg/kg/day from use in the main embryo-fetal study. 2000 mg/kg/day is also considered to be the maximum practical dosage in the rabbit with this material under the conditions of use in this study. It is concluded that the dosage sequence applied in this preliminary study, 0 (Control), 500, 1000 and 2000 mg/kg/day would also be suitable for use in the main embryo-fetal development study.

FIGURE 1

Bodyweight change – group mean values during gestation

Group	:	1	2	3	4
Compound	:	Control	---------- ANS 9801 ----------		
Dosage (mg/kg/day)	:	0	500	1000	2000

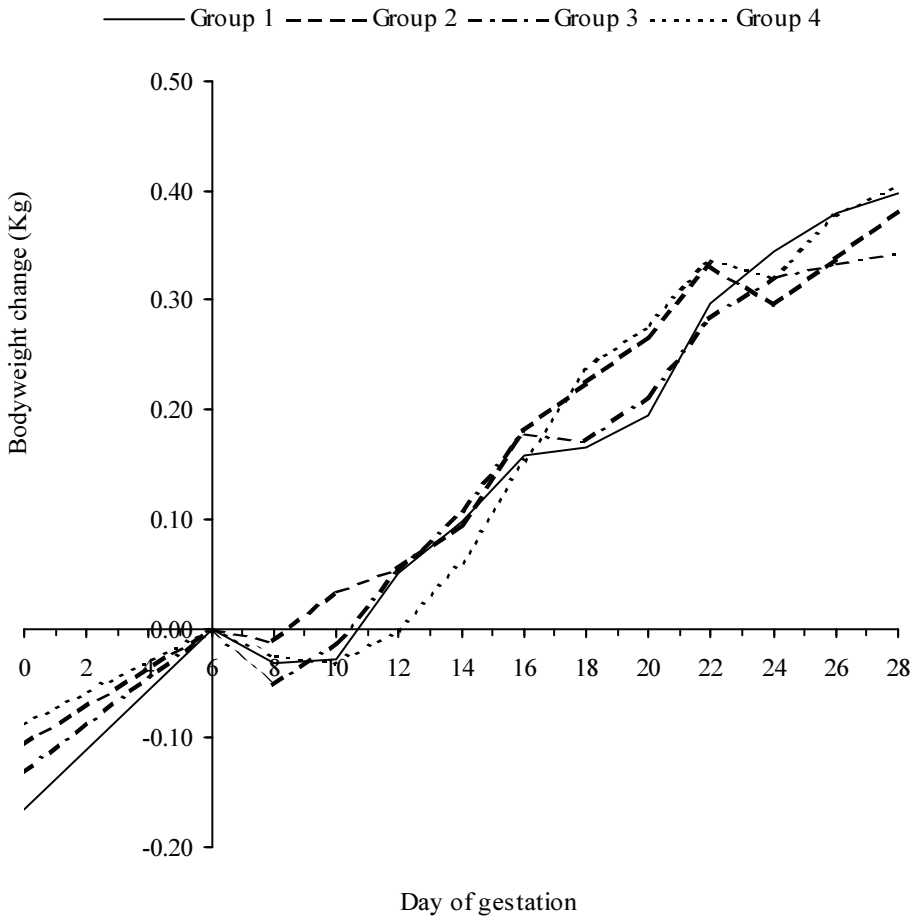

TABLE 1

Summary of adult performance

Group	:	1	2	3	4
Compound	:	Control	---------- ANS 9801 ----------		
Dosage (mg/kg/day)	:	0	500	1000	2000

Category	Group 1	Group 2	Group 3	Group 4
Mated	6	6	6	6
Dosed	6	6	6	6
Killed for humane reasons	0	0	0	1
Aborted	1	1	0	0
Survived to termination	5	5	6	5
Not pregnant	1	0	0	1
With live young at termination	4	5	6	4

TABLE 2

Bodyweight - group mean values (kg) during gestation

Group	:	1	2	3	4
Compound	:	Control	---------- ANS 9801 ----------		
Dosage (mg/kg/day)	:	0	500	1000	2000

Group		Day of gestation												
		0	6	8	10	12	14	16	18	20	22	24	26	28
1	Mean	4.01	4.17	4.14	4.15	4.22	4.27	4.33	4.34	4.37	4.47	4.52	4.55	4.57
	SD	0.31	0.27	0.26	0.25	0.28	0.29	0.21	0.26	0.31	0.29	0.28	0.28	0.26
	n	4	4	4	4	4	4	4	4	4	4	4	4	4
2	Mean	4.19	4.30	4.29	4.33	4.35	4.39	4.48	4.52	4.56	4.63	4.59	4.64	4.68
	SD	0.24	0.23	0.25	0.22	0.24	0.27	0.31	0.39	0.40	0.34	0.33	0.39	0.44
	n	5	5	5	5	5	5	5	5	5	5	5	5	5
3	Mean	4.06	4.19	4.14	4.18	4.24	4.30	4.37	4.37	4.41	4.48	4.51	4.53	4.54
	SD	0.11	0.15	0.17	0.20	0.20	0.22	0.27	0.29	0.27	0.26	0.28	0.31	0.31
	n	6	6	6	6	6	6	6	6	6	6	6	6	6
4	Mean	4.32	4.41	4.39	4.38	4.41	4.47	4.56	4.65	4.69	4.75	4.73	4.79	4.82
	SD	0.17	0.29	0.27	0.24	0.26	0.25	0.25	0.26	0.26	0.31	0.33	0.37	0.37
	n	4	4	4	4	4	4	4	4	4	4	4	4	4

n Number of females with live young on Day 29

TABLE 3

Bodyweight change - group mean values (kg) during gestation

Group	:	1	2	3	4
Compound	:	Control	---------- ANS 9801 ----------		
Dosage (mg/kg/day)	:	0	500	1000	2000

Group		Days of gestation											
		0-6	6-8	6-10	6-12	6-14	6-16	6-18	6-20	6-22	6-24	6-26	6-28
1	Mean	0.17	-0.03	-0.03	0.05	0.10	0.16	0.17	0.20	0.30	0.35	0.38	0.40
	SD	0.05	0.09	0.10	0.15	0.20	0.19	0.21	0.26	0.21	0.19	0.20	0.18
	n	4	4	4	4	4	4	4	4	4	4	4	4
2	Mean	0.11	-0.01	0.03	0.05	0.09	0.18	0.22	0.27	0.33	0.30	0.34	0.38
	SD	0.09	0.07	0.09	0.08	0.07	0.10	0.17	0.18	0.13	0.17	0.24	0.28
	n	5	5	5	5	5	5	5	5	5	5	5	5
3	Mean	0.13	-0.05	-0.01	0.05	0.11	0.18	0.17	0.21	0.28	0.32	0.33	0.34
	SD	0.11	0.06	0.07	0.07	0.10	0.13	0.15	0.14	0.14	0.15	0.19	0.20
	n	6	6	6	6	6	6	6	6	6	6	6	6
4	Mean	0.09	-0.03	-0.03	0.00	0.06	0.15	0.24	0.28	0.34	0.32	0.38	0.41
	SD	0.12	0.08	0.07	0.07	0.14	0.14	0.14	0.18	0.20	0.29	0.32	0.27
	n	4	4	4	4	4	4	4	4	4	4	4	4

n Number of females with live young on Day 29

TABLE 4

Gravid uterine weight, adjusted bodyweight and adjusted bodyweight change – group mean values (kg) on Day 29 of gestation

Group	:	1	2	3	4
Compound	:	Control	---------- ANS 9801 ----------		
Dosage (mg/kg/day)	:	0	500	1000	2000

Group		Bodyweight		Bodyweight gain	Gravid uterus	Adjusted Day 29	Adjusted bodyweight gain
		Day 6	Day 29	Days 6-29	weight	Bodyweight	Days 6-29
1	Mean	4.17	4.60	0.43	0.518	4.08	-0.09
	SD	0.27	0.28	0.19	0.1	0.33	0.17
	n	4	4	4	4	4	4
2	Mean	4.30	4.71	0.41	0.406	4.30	0.00
	SD	0.23	0.50	0.34	0.2	0.46	0.32
	n	5	5	5	5	5	5
3	Mean	4.19	4.54	0.35	0.637	3.91	-0.29
	SD	0.15	0.30	0.21	0.1	0.24	0.16
	n	6	6	6	6	6	6
4	Mean	4.41	4.80	0.39	0.506	4.29	-0.12
	SD	0.29	0.39	0.26	0.2	0.40	0.33
	n	4	4	4	4	4	4

n Number of females with live young on Day 29

TABLE 5

Food consumption – group mean values (g/rabbit) during gestation

Group	:	1	2	3	4
Compound	:	Control	---------- ANS 9801 ----------		
Dosage (mg/kg/day)	:	0	500	1000	2000

Group /sex		Day of gestation													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	Mean	173	185	183	197	183	124	135	130	122	173	122	148	146	144
	SD	33	19	18	13	40	35	80	63	50	52	62	69	42	42
	n	4	4	4	4	4	4	4	4	4	4	4	4	4	4
2	Mean	210	192	188	202	180	157	186	159	177	182	162	184	177	169
	SD	16	24	25	18	54	27	25	20	19	23	33	40	41	39
	n	5	5	5	5	5	5	5	5	5	5	5	5	5	5
3	Mean	211	200	210	196	185	113	137	120	158	169	171	175	165	139
	SD	17	17	29	18	32	32	50	28	37	28	25	51	61	60
	n	6	6	6	6	6	6	6	6	6	6	6	6	6	6
4	Mean	148	209	176	198	229	131	183	129	182	146	166	195	170	174
	SD	75	36	55	36	49	55	28	25	47	67	27	76	44	48
	n	4	4	4	4	4	4	4	4	4	4	4	4	4	4

n Number of females with live young at Day 29 of gestation

TABLE 5 - continued

Food consumption – group mean values (g/rabbit) during gestation

Group	:	1	2	3	4
Compound	:	Control	---------- ANS 9801 ----------		
Dosage (mg/kg/day)	:	0	500	1000	2000

Group /sex		Day of gestation													
		15	16	17	18	19	20	21	22	23	24	25	26	27	28
1	Mean	120	117	115	105	172	204	184	174	157	140	158	127	129	133
	SD	74	74	64	57	57	33	20	30	34	38	18	19	15	19
	n	4	4	4	4	4	4	4	4	4	4	4	4	4	4
2	Mean	201	198	172	162	198	199	190	136	131	118	147	133	141	137
	SD	59	46	88	88	73	31	58	96	76	92	70	67	79	88
	n	5	5	5	5	5	5	5	5	5	5	5	5	5	5
3	Mean	169	148	143	153	162	179	166	151	121	111	101	96	88	87
	SD	71	62	60	67	48	38	40	44	50	61	60	45	46	50
	n	6	6	6	6	6	6	6	6	6	6	6	6	6	6
4	Mean	200	220	208	223	232	214	189	202	146	143	141	116	113	88
	SD	39	19	37	28	37	62	83	65	72	90	91	76	65	86
	n	4	4	4	4	4	4	4	4	4	4	4	4	4	4

n Number of females with live young at Day 29 of gestation

TABLE 6

Water consumption – group mean values (g/rabbit) during gestation

Group	:	1	2	3	4
Compound	:	Control	---------- ANS 9801 ----------		
Dosage (mg/kg/day)	:	0	500	1000	2000

Group		Day of gestation				
		1	8	13	20	26
1	Mean	268	362	560	381	406
	SD	77	109	264	114	226
	n	3	4	4	4	4
2	Mean	279	383	403	465	397
	SD	92	147	177	256	258
	n	2	4	4	4	5
3	Mean	373	366	464	775	375
	SD	112	134	133	218	289
	n	3	6	6	5	6
4	Mean	116	465	575	557	432
	SD	109	221	65	178	130
	n	3	4	4	4	4

n Number of females with live young at Day 29 of gestation

TABLE 7

Litter data – group mean values on Day 29 of gestation

Group	:	1	2	3	4
Compound	:	Control	---------- ANS 9801 ----------		
Dosage (mg/kg/day)	:	0	500	1000	2000

Group		Corpora Lutea	Implantations	Resorptions			Live young			Sex ratio (% M)	Implantation loss (%)	
				Early	Late	Total	Male	Female	Total		Pre-	Post-
1	Mean	11.5	10.8	0.5	2.0	2.5	3.3	5.0	8.3	40.5	5.8	19.6
	SD	4.0	3.8				0.5	1.6	1.7			
	n	4	4	4	4	4	4	4	4	4	4	4
2	Mean	12.4	7.4	0.4	1.2	1.6	2.6	3.2	5.8	33.7	40.7	17.6
	SD	1.7	4.4				2.4	1.3	3.5			
	n	5	5	5	5	5	5	5	5	5	5	5
3	Mean	12.3	11.7	0.2	0.5	0.7	5.3	5.7	11.0	48.2	5.6	5.3
	SD	1.6	2.2				2.3	2.4	2.1			
	n	6	6	6	6	6	6	6	6	6	6	6
4	Mean	13.0	10.8	1.0	1.5	2.5	4.0	4.3	8.3	50.6	19.4	25.8
	SD	3.6	3.9				2.9	3.0	3.6			
	n	4	4	4	4	4	4	4	4	4	4	4

n Number of litters

TABLE 8

Placental, litter and fetal weights – group mean values (g) on Day 29 of gestation

Group	:	1	2	3	4
Compound	:	Control	---------- ANS 9801 ----------		
Dosage (mg/kg/day)	:	0	500	1000	2000

Group		Placental weight	Litter weight	Fetal weights		
				Males	Females	Overall
1	Mean	5.3	319.6	38.8	40.2	39.8
	SD	1.7	42.8	9.8	7.2	7.9
	n	4	4	4	4	4
2	Mean	6.0	235.0	41.1	42.6	42.7
	SD	1.2	143.5	8.0	8.8	8.5
	n	5	5	4	5	5
3	Mean	5.4	426.6	40.1	38.6	39.1
	SD	0.5	63.6	5.0	4.2	4.0
	n	6	6	6	6	6
4	Mean	5.6	297.3	39.7	41.0	40.0
	SD	2.0	91.7	12.4	13.9	12.9
	n	4	4	4	4	4

n Number of litters

APPENDIX 1

Summary of mortality and abortion

Group	:	1	2	3	4
Compound	:	Control	---------- ANS9801 ----------		
Dosage (mg/kg/day)	:	0	500	1000	2000

Group	Animal number	Status	Clinical signs observed (on Day(s) of gestation)	Necropsy findings
1	1	Aborted Day 20 of gestation	Red staining on cage tray paper (20). Small amount of resorbing fetal material and fetal portion of placenta found on cage tray (20).	Yellow staining on paws. **Left uterine horn:** No implantations **Right uterine horn:** 1 implantation, only maternal portion of placenta present
2	10	Aborted Day 20 of gestation	Little water drunk and few faeces (4) 1 grossly normal, dead fetus found in cage tray (20).	Yellow staining on all paws. **Left uterine horn:** 1 implantation site containing placenta only **Right uterine horn:** No implantations
4	24	Killed for humane reasons, Day 21 of gestation	Green staining on cage tray paper (10-21) and on whole body (16-21). Few (5, 10, 14-21) and loose (16-21) faeces. Swollen and red ano-genital area (19-21).	Green staining on all paws and ventral abdomen. **Labia:** appear slightly reddened and pronounced **Caecum:** contained large amount of gas and dark fluid **Urinary bladder:** contents green **Uterus:** 12 implantations – all fetuses grossly normal. Maternal portions of all placentae appeared slightly enlarged in relation to fetal portion.

APPENDIX 2

Clinical signs – individual observations

Group 1: Control

Group	Animal number	Clinical signs observed (Days after mating)
1	1 AB (20)	*See summary of mortality and abortion* (Appendix 1)
	2	-
	3	Orange staining on cage tray paper (22-29) Loose faeces (10, 20)
	4 NP	Hairloss on ventral body surface (7-20, 23-29)
	5	Yellow staining on tail (22-29)
	6	Decreased faecal output (17-21)

- No clinical signs observed
AB (n) Animal aborted (Day of gestation)
NP Not pregnant

APPENDIX 2 - continued

Clinical signs – individual observations

Group 2: ANS 9801: 500mg/kg/day

Group	Animal number	Clinical signs observed (Days after mating)
2	7	Green staining on cage tray paper (9-29) Yellow staining on all paws (19-29)
	8	Green (19-27) and green/pink (28-29) staining on cage tray paper
	9	Red/purple staining on perigenital region, ventral body surface, tail and cage tray paper (18-19) Green staining on all paws, perigenital region, ventral body surface, tail (20-28) and cage tray paper (20-29) Yellow/ green staining on all paws and tail (29) Decreased faecal output (18-20, 23-29) and loose faeces (18-20)
	10 AB (20)	*See summary of mortality and abortion* (Appendix 1)
	11	Partially absent pinna from Day 0 Red (24-26) and green (27-29) staining on cage tray paper Decreased faecal output (23-26)
	12	Yellow staining on hindpaws (7-29) Green (9-17) and pink/green (18-23) staining on cage tray paper

AB (n) Animal aborted (Day of gestation)

APPENDIX 2 -continued

Clinical signs – individual observations

Group 3: ANS 9801: 1000mg/kg/day

Group	Animal number	Clinical signs observed (Days after mating)
3	13	Green staining on all paws (19-29) and cage tray paper (10-29)
		Urine green in colour (7-9)
	14	Green/red (9-24) and green (25-29) staining on cage tray paper
		Yellow/green (15-24) and yellow (25-29) staining on all paws
		Urine green in colour (7-8)
	15	Green (9-16, 20-22) and green/red (17-19) staining on cage tray paper
		Yellow staining on all paws (20-29) and tail (20-27)
		Green staining on tail (28-29)
		Decreased faecal output (16-21)
		Loose (17-18) and pale (19-22) faeces
		Urine green and purple in colour (7-8)
	16	Green staining on all paws (9-29), head, ventral body surface and tail (16-29)
		Green (9-10, 16-20) and green/purple (11-15, 21-29) staining on cage tray paper
		Urine green in colour (7-9)
	17	Green staining on cage tray paper (10-29)
		Brown staining on tail (29)
	18	Green staining on all paws (15-29), tail (19-29) and cage tray paper (8-29)
		Faeces grey (25-28), reduced in output (25-29) and pale (29)

APPENDIX 2 -continued

Clinical signs – individual observations

Group 4: ANS 9801: 2000mg/kg/day

Group	Animal number	Clinical signs observed (Days after mating)
4	19	Brown staining on perigenital region (7-15)
		Green staining on tail (8-29), all paws and perigenital region (16-29) and cage tray paper (8-29)
		Pale faeces (17-29)
	20	Brown staining on hindpaws and tail (7-17)
		Green staining on all paws, tail (18-29) and cage tray paper (8-29)
		Pale faeces (17-22, 25-29)
	21	Green (9-17,29) and green/red (18-28) staining on cage tray paper
		Yellow staining on tail and muzzle (29)
		Pale faeces (19-29), decreased faecal output (22-29)
	22	Green (16-24), green/red (9-15) and green/purple (25-29) staining on cage tray paper
		Pale faeces (28-29)
		Hairloss on ventral (7-10) and dorsal (28-29) body surfaces
		Aqueous discharge left eye (22-29)
	23 NP	Green staining on all paws and tail (19-24)
		Yellow staining on all paws (25-29)
		Green (8-24) and green/purple (25-29) staining on cage tray paper
		Bruising on left pinna (7-10, 28-29)
	24 HK (21)	*See summary of mortality and abortion* (Appendix 1)

NP Not pregnant
HK (n) Killed for humane reasons (Day after mating)

APPENDIX 3

Bodyweight – individual values (kg) after mating

Group 1: Control

Animal number	Day after mating												
	0	6	8	10	12	14	16	18	20	22	24	26	28
1 AB (20)	4.14	4.17	4.08	4.15	4.19	4.26	4.33	4.33	4.22				
2	3.65	3.88	3.87	3.95	4.07	4.16	4.23	4.24	4.23	4.30	4.35	4.41	4.41
3	4.34	4.48	4.50	4.50	4.64	4.70	4.64	4.73	4.81	4.90	4.93	4.97	4.95
4 NP	4.09	4.13	4.14	4.16	4.21	4.27	4.25	4.31	4.35	4.49	4.59	4.60	4.59
5	3.87	4.04	4.07	4.01	4.02	4.10	4.27	4.22	4.33	4.41	4.43	4.45	4.50
6	4.17	4.29	4.12	4.12	4.16	4.12	4.18	4.16	4.10	4.27	4.36	4.38	4.42

Group 2: ANS 9801: 500mg/kg/day

Animal number	Day after mating												
	0	6	8	10	12	14	16	18	20	22	24	26	28
7	4.00	4.16	4.08	4.10	4.18	4.17	4.22	4.23	4.30	4.33	4.34	4.41	4.41
8	4.17	4.26	4.22	4.30	4.24	4.34	4.57	4.62	4.63	4.70	4.78	4.90	4.97
9	4.14	4.11	4.19	4.30	4.30	4.27	4.22	4.13	4.14	4.32	4.19	4.10	4.08
10 AB (20)	3.59	3.67	3.54	3.66	3.71	3.77	3.82	3.89	3.70				
11	4.61	4.70	4.73	4.70	4.77	4.86	4.96	5.11	5.19	5.14	5.02	5.09	5.16
12	4.04	4.26	4.21	4.26	4.27	4.32	4.42	4.52	4.56	4.66	4.64	4.68	4.78

AB(n) Animal aborted (Day of gestation)
NP Not pregnant

APPENDIX 3 - continued

Bodyweight – individual values (kg) after mating

Group 3: ANS 9801: 1000mg/kg/day

Animal number	Day after mating												
	0	6	8	10	12	14	16	18	20	22	24	26	28
13	3.96	4.27	4.25	4.34	4.41	4.47	4.55	4.50	4.55	4.59	4.59	4.59	4.60
14	4.08	4.15	4.08	4.08	4.15	4.22	4.32	4.35	4.42	4.58	4.64	4.70	4.72
15	3.97	3.96	3.85	3.89	3.92	3.90	3.88	3.84	3.89	3.97	4.02	4.11	4.13
16	4.08	4.21	4.13	4.13	4.24	4.36	4.46	4.43	4.45	4.49	4.61	4.55	4.63
17	4.25	4.42	4.33	4.45	4.49	4.52	4.67	4.70	4.69	4.74	4.83	4.96	4.93
18	4.03	4.15	4.21	4.19	4.25	4.33	4.35	4.37	4.43	4.49	4.39	4.25	4.21

Group 4: ANS 9801: 2000mg/kg/day

Animal number	Day after mating												
	0	6	8	10	12	14	16	18	20	22	24	26	28
19	4.51	4.74	4.61	4.62	4.66	4.61	4.70	4.78	4.76	4.84	4.69	4.72	4.85
20	4.31	4.40	4.39	4.35	4.38	4.50	4.63	4.73	4.80	4.87	4.91	5.01	5.01
21	4.10	4.04	4.01	4.06	4.05	4.11	4.19	4.26	4.31	4.29	4.28	4.29	4.29
22	4.37	4.46	4.53	4.48	4.54	4.66	4.73	4.82	4.88	4.99	5.04	5.13	5.11
23 NP	4.62	4.76	4.70	4.69	4.69	4.68	4.62	4.73	4.83	4.98	4.99	5.01	4.99
24 HK (21)	4.23	4.24	4.15	4.18	4.22	4.14	3.88	3.76	3.84				

NP Not pregnant
HK(n) Animal killed for humane reasons (Day of gestation)

APPENDIX 4

Gravid uterine weight, adjusted bodyweight and adjusted bodyweight change – individual values (kg) on Day 29 of gestation

Group 1: Control

Animal number	Bodyweight		Bodyweight gain Days 6-29	Gravid uterus weight	Adjusted Day 29 bodyweight	Adjusted bodyweight gain change Day 6-29
	Day 6	Day 29				
1 AB (20)						
2	3.88	4.43	0.55	0.621	3.81	-0.07
3	4.48	5.02	0.54	0.454	4.57	0.09
4 NP						
5	4.04	4.51	0.47	0.521	3.99	-0.05
6	4.29	4.44	0.15	0.476	3.96	-0.33

Group 2: ANS 9801: 500mg/kg/day

Animal number	Bodyweight		Bodyweight gain Days 6-29	Gravid uterus weight	Adjusted Day 29 bodyweight	Adjusted bodyweight gain change Day 6-29
	Day 6	Day 29				
7	4.16	4.40	0.24	0.305	4.09	-0.07
8	4.26	5.04	0.78	0.744	4.30	0.04
9	4.11	4.02	-0.09	0.375	3.64	-0.47
10 AB (20)						
11	4.70	5.25	0.55	0.489	4.76	0.06
12	4.26	4.82	0.56	0.118	4.70	0.44

AB(n) Animal aborted (Day of gestation)
NP Not pregnant

APPENDIX 4 - continued

Gravid uterine weight, adjusted bodyweight and adjusted bodyweight change – individual values (g) on Day 29 of gestation

Group 3: ANS 9801: 1000mg/kg/day

Animal number	Bodyweight		Bodyweight gain Days 6-29	Gravid uterus weight	Adjusted Day 29 bodyweight	Adjusted bodyweight gain change Day 6-29
	Day 6	Day 29				
13	4.27	4.66	0.39	0.614	4.05	-0.22
14	4.15	4.73	0.58	0.606	4.12	-0.03
15	3.96	4.20	0.24	0.618	3.58	-0.38
16	4.21	4.61	0.40	0.665	3.94	-0.27
17	4.42	4.91	0.49	0.812	4.10	-0.32
18	4.15	4.15	0.00	0.504	3.65	-0.50

Group 4: ANS 9801: 2000mg/kg/day

Animal number	Bodyweight		Bodyweight gain Days 6-29	Gravid uterus weight	Adjusted Day 29 bodyweight	Adjusted bodyweight gain change Day 6-29
	Day 6	Day 29				
19	4.74	4.87	0.13	0.551	4.32	-0.42
20	4.40	5.02	0.62	0.281	4.74	0.34
21	4.04	4.23	0.19	0.467	3.76	-0.28
22	4.46	5.06	0.60	0.725	4.34	-0.12
23 NP						
24 HK (21)						

NP Not pregnant
HK(n) Animal killed for humane reasons (Day of gestation)

APPENDIX 5

Food consumption – individual values (g/rabbit) after mating

Group 1: Control

Animal number	Day after mating													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1 AB (20)	210	201	178	192	141	97	167	151	180	223	189	265	212	174
2	194	201	202	208	212	153	182	168	168	190	170	197	198	171
3	130	170	163	198	206	151	198	190	149	232	181	218	146	117
4 NP	212	214	222	202	227	171	221	149	230	225	213	228	223	189
5	164	167	172	179	188	112	139	111	55	107	70	95	147	187
6	203	200	193	202	124	79	20	49	114	162	68	82	94	100

Animal number	Day after mating													
	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1 AB (20)	217	224	203	114	145									
2	168	157	145	144	178	187	169	157	145	139	143	102	126	116
3	114	144	178	154	229	252	211	210	205	192	183	147	120	158
4 NP	204	170	227	218	229	256	245	231	237	202	249	251	220	250
5	180	160	110	92	187	198	185	185	151	128	155	125	119	136
6	18	6	28	29	94	178	170	143	126	102	150	132	150	122

AB(n) Animal aborted (Day of gestation)
NP Not pregnant

APPENDIX 5 - continued

Food consumption – individual values (g/rabbit) after mating

Group 2: ANS 9801: 500mg/kg/day

Animal number	Day after mating													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
7	182	179	160	186	113	113	158	125	150	173	160	152	160	149
8	220	171	165	193	149	162	202	163	175	147	121	156	166	195
9	213	181	195	211	228	161	194	168	204	199	178	171	148	112
10 AB (20)	122	143	50	188	114	117	142	135	174	221	175	204	216	174
11	210	231	218	230	243	187	216	175	178	206	208	249	249	211
12	224	196	202	190	168	161	162	165	179	185	143	191	162	176

Animal number	Day after mating													
	15	16	17	18	19	20	21	22	23	24	25	26	27	28
7	124	134	140	145	174	172	182	182	154	144	158	97	153	139
8	251	211	230	228	214	221	209	203	178	168	176	171	131	143
9	152	173	35	15	85	179	210	42	31	1	39	49	32	2
10 AB (20)	159	155	156	127	102									
11	252	256	257	216	262	181	97	23	74	48	131	126	134	153
12	224	215	197	206	256	244	253	228	217	227	229	223	255	248

AB(n) Animal aborted (Day of gestation)

APPENDIX 5 – continued

Food consumption – individual values (g/rabbit) after mating

Group 3: ANS 9801: 1000mg/kg/day

Animal number	Day after mating													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
13	221	208	256	216	204	125	167	140	216	214	202	189	184	149
14	212	192	216	199	193	132	109	76	149	127	166	200	219	189
15	191	171	167	164	146	54	67	95	141	168	132	88	50	26
16	235	208	202	187	151	99	159	123	105	164	170	181	182	165
17	191	221	215	205	187	127	116	136	175	173	164	152	150	124
18	215	198	203	205	231	140	206	147	162	166	194	239	206	179

Animal number	Day after mating													
	15	16	17	18	19	20	21	22	23	24	25	26	27	28
13	217	157	192	193	208	214	169	154	94	93	95	83	94	131
14	214	172	159	179	128	207	235	228	200	206	185	167	119	97
15	32	34	29	23	99	144	141	138	129	133	136	126	118	146
16	199	193	130	152	148	136	159	169	148	102	47	84	128	89
17	150	130	172	160	165	154	118	106	97	112	122	81	58	46
18	199	203	176	208	224	219	174	113	55	18	22	36	8	13

APPENDIX 5 – continued

Food consumption – individual values (g/rabbit) after mating

Group 4: ANS 9801: 2000mg/kg/day

Animal number	Day after mating													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
19	246	253	227	242	258	148	187	139	156	159	138	110	115	110
20	163	190	193	211	202	64	197	100	151	61	191	291	218	227
21	109	172	186	163	174	118	142	157	171	142	148	169	157	175
22	73	222	98	174	280	195	205	119	251	223	188	208	189	183
23 NP	264	179	281	247	214	137	189	198	196	216	201	228	77	141
24 HK (21)	209	177	79	31	155	102	144	140	161	176	129	55	10	3

Animal number	Day after mating													
	15	16	17	18	19	20	21	22	23	24	25	26	27	28
19	161	198	191	202	193	186	212	115	77	61	60	88	131	132
20	253	243	222	254	256	270	241	274	220	244	245	228	194	186
21	188	213	166	197	209	139	65	210	91	72	70	60	42	28
22	197	225	251	238	270	259	237	207	194	194	190	88	85	5
23 NP	233	228	261	250	296	256	276	280	237	216	249	215	214	219
24 HK (21)		21	16	50	6	3								

NP Not pregnant
HK(n) Animal killed for humane reasons (Day of gestation)

APPENDIX 6

Water consumption – individual values (g/rabbit) after mating

Group 1: Control

Animal number	Day after mating				
	1	8	13	20	26
1 AB (20)	424	322	507		
2	320	292	438	276	232
3	180	487	776	446	423
4 NP	@	551	412	334	474
5	@	416	780	508	718
6	305	251	244	293	249

Group 2: ANS 9801: 500mg/kg/day

Animal number	Day after mating				
	1	8	13	20	26
7	214	221	249	223	198
8	344	372	387	332	339
9	@	362	321	494	130
10 AB (20)	@	639	@		
11	@	@	@	@	571
12	@	578	654	810	747

AB(n) Animal aborted (Day of gestation)
NP Not pregnant
@ Water bottle empty – value excluded

APPENDIX 6 - continued

Water consumption – individual values (g/rabbit) after mating

Group 3: ANS 9801: 1000mg/kg/day

Animal number	Day after mating				
	1	8	13	20	26
13	502	280	444	402	222
14	305	589	621	795	910
15	313	274	344	966	511
16	@	322	557	887	224
17	@	259	279	@	200
18	@	469	538	824	182

Group 4: ANS 9801: 2000mg/kg/day

Animal number	Day after mating				
	1	8	13	20	26
19	@	586	502	664	331
20	239	143	545	550	521
21	76	630	600	309	566
22	32	499	652	705	310
23 NP	670	556	183	569	528
24 HK (21)	@	358	91	119	

NP Not pregnant
HK(n) Animal killed for humane reasons (Day of gestation)
@ Water bottle empty – value excluded

APPENDIX 7

Necropsy findings – individual observations

Group 1: Control

Animal number	Necropsy observations
1 AB (20)	*See Summary of mortality and abortion* (Appendix 1)
2	Yellow staining on all paws
3	Yellow staining on all paws Bruising on left pinna
4 NP	Yellow staining on all paws and tail Hairloss on ano-genital region
5	Yellow staining on all paws and tail
6	Yellow staining on all paws and tail

AB(n) Animal aborted (Day of gestation)
NP Not pregnant

APPENDIX 7 - continued

Necropsy findings – individual observations

Group 2: 500 mg/kg/day

Animal number	Necropsy observations
7	Yellow staining on all paws
8	Yellow/green staining on all paws and tail
9	Yellow staining on all paws **Urinary bladder**: contents dark green
10 AB(20)	See Summary of mortality and abortion (Appendix 1)
11	Yellow staining on all paws and tail **Urinary bladder**: contents dark green **Caecum**: contents dark
12	Yellow staining on all paws

AB(n) Animal aborted (Day of gestation)

APPENDIX 7 - continued

Necropsy findings – individual observations

Group 3: 1000 mg/kg/day

Animal number	Necropsy observations
13	Yellow/green staining on all paws
14	Yellow/green staining on all paws
15	Yellow/green staining on all paws and tail
16	Green staining on all paws and tail
17	Yellow staining on all paws Grey staining on tail **Liver**: pale with accentuated lobular pattern **Gall bladder**: enlarged
18	Green staining on all paws and tail **Caecum:** large amount of dark gaseous fluid present

APPENDIX 7 - continued

Necropsy findings – individual observations

Group 4: 2000 mg/kg/day

Animal number	Necropsy observations
19	Green staining on all paws and around urino-genital area **Lungs**: numerous dark areas on all lobes
20	Green/yellow staining on all paws
21	Yellow staining on all paws and muzzle Brown/green staining on tail **Caecum**: large amount of gas and dark fluid present
22	Yellow staining on all paws Green/brown staining on muzzle and tail Matted fur around left eye Hairloss on dorsal cervical region **Lungs**: punctate dark foci on all lobes **Liver**: accentuated lobular pattern **Stomach**: contents reduced **Caecum**: distended with gas and dark fluid contents **Urinary bladder**: dark contents
23 NP	Yellow staining on all paws
24 HK (21)	See Summary of mortality and abortion (Appendix 1)

NP Not pregnant
HK(n) Killed for reasons of animal welfare (Day of gestation)

APPENDIX 8

Litter data – individual litter values on Day 29 of gestation

Group 1: Control

Animal number	Corpora lutea	Implants	Resorptions			Live young			Sex ratio (% M)	Implantation loss (%)	
			Early	Late	Total	Male	Female	Total		Pre-	Post-
1 AB (20)											
2	14	12	2	1	3	4	5	9	44.4	14.3	25.0
3	6	6	0	0	0	3	3	6	50.0	0.0	0.0
4 NP											
5 U	15	15	0	5	5	3	7	10	30.0	0.0	33.3
6	11	10	0	2	2	3	5	8	37.5	9.1	20.0

Group 2: 500 mg/kg/day

Animal Number	Corpora lutea	Implants	Resorptions			Live young			Sex ratio (% M)	Implantation loss (%)	
			Early	Late	Total	Male	Female	Total		Pre-	Post-
7	11	5	1	0	1	1	3	4	25.0	54.5	20.0
8	11	12	1	1	2	6	4	10	60.0	0.0 @	16.7
9	12	10	0	4	4	2	4	6	33.3	16.7	40.0
10 AB (20)											
11	15	9	0	1	1	4	4	8	50.0	40.0	11.1
12 U	13	1	0	0	0	0	1	1	0.0	92.3	0.0

AB (n) Animal Aborted (Day of gestation)
NP Not pregnant
U Unilateral implantation
@ Number of implantations exceeds corpora lutea count, pre-implantation loss assumed to be zero

APPENDIX 8 - continued

Litter data – individual litter values on Day 29 of gestation

Group 3: ANS 9801: 1000mg/kg/day

Animal number	Corpora lutea	Implants	Resorptions			Live young			Sex ratio	Implantation loss (%)	
			Early	Late	Total	Male	Female	Total	(% M)	Pre-	Post-
13	12	12	1	1	2	6	4	10	60.0	0.0	16.7
14	12	9	0	0	0	1	8	9	11.1	25.0	0.0
15	13	13	0	2	2	5	6	11	45.5	0.0	15.4
16	12	11	0	0	0	8	3	11	72.7	8.3	0.0
17	15	15	0	0	0	6	9	15	40.0	0.0	0.0
18	10	10	0	0	0	6	4	10	60.0	0.0	0.0

Group 4: ANS 9801: 2000mg/kg/day

Animal number	Corpora lutea	Implants	Resorptions			Live young			Sex ratio	Implantation loss (%)	
			Early	Late	Total	Male	Female	Total	(% M)	Pre-	Post-
19	16	14	0	3	3	6	5	11	54.5	12.5	21.4
20	8	5	2	0	2	2	1	3	66.7	37.5	40.0
21	13	12	2	0	2	7	3	10	70.0	7.7	16.7
22	15	12	0	3	3	1	8	9	11.1	20.0	25.0
23 NP											
24 HK (21)											

NP Not pregnant
HK (n) Animal killed for human reasons (Day of gestation)

APPENDIX 9

Placental, litter and fetal weights – individual litter values (g) on Day 29 of gestation

Group 1: Control

Animal Number	Placental weights		Litter weight	Fetal weights					
				Males		Females		Total	
	Mean	SD		Mean	SD	Mean	SD	Mean	SD
1 AB									
2	5.0	0.7	382.4	44.4	1.4	41.0	2.9	42.5	2.8
3	7.6	0.7	296.4	49.7	2.6	49.1	2.5	49.4	2.3
4 NP									
5 U	3.4	0.7	310.7	29.6	10.0	31.7	6.0	31.1	6.9
6	5.0	0.6	288.9	31.5	4.7	38.9	3.6	36.1	5.3

Group 2: 500 mg/kg/day

Animal Number	Placental weights		Litter weight	Fetal weights					
				Males		Females		Total	
	Mean	SD		Mean	SD	Mean	SD	Mean	SD
7	6.1	0.4	195.1	50.6		48.2	1.9	48.8	1.9
8	5.6	0.9	431.1	41.6	1.5	45.4	2.2	43.1	2.6
9	5.0	0.7	182.9	31.0	3.5	30.2	3.0	30.5	2.8
10 AB (20)									
11	5.4	0.6	313.8	41.4	1.3	37.1	1.5	39.2	2.6
12 U	8.0		52.1			52.1		52.1	

AB (n) Animal aborted (Day of gestation)
NP Not pregnant
U Unilateral implantation

APPENDIX 9 – continued

Placental, litter and fetal weights – individual litter values (g) on Day 29 of gestation

Group 3: ANS 9801: 1000mg/kg/day

Animal Number	Placental weights		Litter weight	Fetal weights					
				Males		Females		Total	
	Mean	SD		Mean	SD	Mean	SD	Mean	SD
13	5.0	0.4	408.8	41.4	3.0	40.2	3.0	40.9	2.9
14	5.4	0.6	407.3	48.9		44.8	2.7	45.3	2.9
15	5.3	1.0	406.3	35.8	5.6	37.9	5.7	36.9	5.5
16	6.0	0.9	454.1	41.5	3.3	40.7	4.2	41.3	3.4
17	5.9	1.1	536.0	37.5	5.1	34.6	5.3	35.7	5.2
18	4.9	0.7	346.8	35.5	9.0	33.4	3.3	34.7	7.0

Group 4: ANS 9801: 2000mg/kg/day

Animal Number	Placental weights		Litter weight	Fetal weights					
				Males		Females		Total	
	Mean	SD		Mean	SD	Mean	SD	Mean	SD
19	3.5	0.4	325.5	29.9	2.7	29.2	4.0	29.6	3.2
20	8.2	0.9	170.8	55.5	1.6	59.9		56.9	2.8
21	4.5	1.0	303.7	29.7	7.9	31.9	7.3	30.4	7.4
22	6.3	0.8	389.0	43.8		43.2	4.0	43.2	3.8
23 NP									
24 HK (21)									

NP Not pregnant
HK (n) Animal killed for humane reasons (Day of gestation)

APPENDIX 10

Fetal necropsy findings – individual observations

Group	:	1	2	3	4
Compound	:	Control	---------- ANS 9801 ----------		
Dosage (mg/kg/day)	:	0	500	1000	2000

Group	Dam number	Within litter fetal identification #	Necropsy observations
1	6	M6 (L)	**Liver:** Pale areas on right and left lobes
3	13	M3 (R)	Persistent left posterior cardinal vein
	17	F4 (R) M5 (R)	Haemorrhage on gall bladder Haemorrhage on gall bladder
4	21	M1 (R)	**Abdomen:** Pale areas, large amount of free cloudy fluid **Heart and kidneys:** Pale **Lungs:** All lobes congested **Caecum and rectum:** Devoid of contents **Liver:** Numerous punctate dark areas on surface of all lobes
	22	F1 (L) M3 (R) F4 (R)	Haemorrhage on gall bladder Haemorrhage on gall bladder Punctate dark area on palate

* Only fetuses with observations are presented

\# Within litter identification: Male/Female, n = uterine position (Left or Right uterine horn)

ADDENDUM 1

Certificate of analysis of ANS9801

DATE: 2001/8/7

CERTIFICATE OF ANALYSIS

Item Name: ANS9801 Drug Substance

Lot Number: 000825

Test item		Specifications		Result
Description		A white to yellow powder		A white powder
Identification	IR	Same as Reference Spectrum..		Same as Reference Spectrum
Purity (HPLC)	Related substance	RRT 0.27 (Aspartame)	≦1.5%	0.15%
		Other related substance	≦1.0% each	0.20%
		Total related substance	≦4.0%	0.69%
Water		2.5～5.0%		3.99%
Content (Titration)		95%～105%		99.9%

Storage condition: ANS9801 Drug Substance should be kept in a tight container at a room temperature.

Manufacturing Date: August 25, 2000

Expiration Date: August 25, 2003

This is to certify that the information listed above is a true and accurate copy of the data obtained for the material identified.

AJINOMOTO CO., INC.

Tomoya Yamamoto

Tomoya Yamamoto
Deputy General Manager
Development Research Laboratories
Pharmaceutical Laboratories

ADDENDUM 2

Toxicokinetic report

NOTICE

In this study concentrations of ANS9801 and ANS9801-acid in rabbit plasma were measured using a liquid chromatographic tandem mass spectrometric method (LC-MS/MS) previously validated at Huntingdon Life Sciences and documented in Report No. AJO170/014335. However, in recent investigations, it was revealed that methanol or hydrochloric acid used in the extraction procedure lead to re-esterification of ANS9801-acid to ANS9801. As a result of re-esterification, a maximum of 5% of ANS9801-acid is converted to ANS9801 (*Cf.* AJO184/034042 and AJO193/042943). In dog plasma, hydrolysis of the sulphate conjugate of ANS9801-acid is also caused by hydrochloric acid and most of the sulphate conjugate is converted to ANS9801-acid (*Cf.* AJO196/034055). It is a possibility that this phenomenon also took place in rabbit plasma if sulphate conjugate was produced.

Due to these unexpected reactions, these assays have generally over-estimated the amount of ANS9801 and may have over-estimated the amount of ANS9801-acid in vivo at many time points. However, the toxicokinetic data give good qualitative evidence of exposure to ANS9801 and ANS9801-acid.

TOXICOKINETIC REPORT

ANS 9801

PRELIMINARY EMBRYO-FETAL TOXICITY STUDY

IN THE RABBIT BY ORAL GAVAGE ADMINISTRATION

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SUMMARY

This report describes the quantification of ANS 9801 and ANS 9801-acid in plasma samples of Female New Zealand white Rabbits following dietary administration of ANS 9801 for 22 days as described in study protocol AJO/183.

Blood samples were taken on Day 6 and Day 27 of pregnancy (Days 1 and 22 of treatment) during an embryo-foetal toxicity study, in order to assess the systemic exposure of pregnant female rabbits to ANS 9801 and its metabolite ANS 9801-acid, following daily oral gavage administration of ANS 9801 at dose levels of 500, 1000 and 2000 mg/kg/day from Day 6 to Day 27 after mating. Plasma concentrations of ANS 9801 and ANS 9801-acid (both measured as anhydrous bases) in samples taken up to 24 hours post-dose were measured by a validated liquid chromatographic - tandem mass spectrometric (LC-MS/MS) method.

Maximum mean plasma concentrations (C_{max}) of ANS 9801 and the areas under the mean plasma ANS 9801 concentration-time curves estimated up to 24 hours post-dose (AUC_{24}) on Day 6 and Day 27 after mating are summarised below:

Dose level	C_{max} (ng/ml)		AUC_{24} (ng.h/ml)	
(mg/kg/day)	Day 6	Day 27	Day 6	Day 27
500	944	847	4982	3906
1000	714	1178	3737	11594
2000	1070	2980	8382	22367

The times at which the maximum mean plasma concentrations occurred (T_{max}) were generally 0.5 hours post-dose, and in the range 0.5 to 1 hour, indicating that absorption was rapid.

The mean plasma concentrations of ANS 9801, at 24 hours post-dose (C_{24}), were quantifiable in all dose groups on Day 6 and Day 27 after mating, therefore, animals were continuously exposed to quantifiable concentrations of ANS 9801 during a dosing interval.

The rate of systemic exposure of pregnant female rabbits to ANS 9801, characterised by C_{max}, was similar at each dose level on Day 6 after mating. On Day 27, the C_{max} values increased with increasing dose over the dose range 500 to 2000 mg/kg/day, however, these increases were less than the proportionate dose increment. Overall, there was statistically significant evidence of non-proportionality (p=0.025).

The extent of systemic exposure of pregnant female rabbits to ANS 9801, characterised by AUC_{24}, was similar at the 500 and 1000 mg/kg/day dose levels on Day 6 after mating, but increased at the 2000 mg/kg/day dose level. However, this increase was *ca* 58% lower than the proportionate dose increment (compared with the value at the 500 mg/kg/day dose level), and there was statistically significant evidence of non-proportionality (p<0.001). On Day 27, the AUC_{24} values increased with increasing dose over the dose range 500 to 2000 mg/kg/day. These increases were greater than the proportionate dose increment, and there was statistically significant evidence of non-proportionality (p=0.002). At the highest dose level (2000 mg/kg/day) the AUC_{24} values on Day 27 were *ca* 1.4-fold higher, than those values predicted from a linear relationship, however, this small departure from linearity was probably not of toxicological importance.

After repeated oral doses (Day 27) the rate (C_{max}) of systemic exposure of pregnant female rabbits to ANS 9801 was similar to that after a single dose (Day 6) at the lowest dose level (500 mg/kg/day). At the two higher dose levels (1000 and 2000 mg/kg/day), however, the C_{max} values were higher after repeated oral doses than those values after a single dose and overall these differences were

statistically significant (p=0.031). The extent (AUC_{24}) of systemic exposure of pregnant female rabbits to ANS 9801 at the lowest dose level (500 mg/kg/day) was lower than that after a single dose (Day 6) and this difference was statistically significant (p=0.034). At the two higher dose levels (1000 and 2000 mg/kg/day) the AUC_{24} values were higher after repeated oral doses than those values after a single dose (Day 6), and these differences were statistically significant (p<0.001). The accumulation ratios were greater than one at the 1000 and 2000 mg/kg/day dose levels, indicating that accumulation occurred after repeated oral administration of ANS 9801 at these dose levels.

Maximum mean plasma concentrations (C_{max}) of ANS 9801-acid and the areas under the mean plasma ANS 9801-acid concentration-time curves estimated up to 24 hours post-dose (AUC_{24}) on Day 6 and Day 27 after mating are summarised below:

Dose level	**C_{max} (ng/ml)**		**AUC_{24} (ng.h/ml)**	
(mg/kg/day)	**Day 6**	**Day 27**	**Day 6**	**Day 27**
500	10513	14338	151896	147414
1000	13694	21382	117112	268999
2000	18923	30400	274417	486206

The times at which the maximum mean plasma concentrations occurred (T_{max}) were generally 1 hour post-dose, and in the range 0.5 to 1 hour, indicating that metabolism from ANS 9801 to its major metabolite ANS 9801-acid, was rapid.

The mean plasma concentrations of ANS 9801-acid at 24 hours post-dose (C_{24}) were quantifiable in all dose groups on Day 6 and Day 27 after mating, therefore, animals were continuously exposed to quantifiable concentrations of ANS 9801-acid during a dosing interval.

The rate and extent of systemic exposure of pregnant female rabbits to ANS 9801-acid, characterised by C_{max} and AUC_{24} respectively, generally increased with increasing dose over the dose range 500 to 2000 mg/kg/day on Day 6 and Day 27 after mating. These increases were less than the proportionate dose increment, and there was statistically significant evidence of non-proportionality (p≤0.030) with the exception of the AUC_{24} values on Day 27 which did not reach statistical significance (p=0.31). Overall, the C_{max} and AUC_{24} values at the highest dose level (2000 mg/kg/day) were *ca* 51% and *ca* 36% lower, respectively, than those values predicted from a linear relationship.

After repeated oral doses of ANS 9801 (Day 27) the rate (C_{max}) of systemic exposure of pregnant female rabbits to ANS 9801-acid was *ca* 1.4 to 1.6-fold higher than those values after a single dose and these differences were statistically significant (p=0.038). The extent (AUC_{24}) of systemic exposure to ANS 9801-acid at the lowest dose level (500 mg/kg/day) on Day 27 after mating was similar to that after a single dose (Day 6), and there was no statistically significant evidence for a time (day of sampling) related difference (p=0.78). However, at the two higher dose levels (1000 and 2000 mg/kg/day) the AUC_{24} values on Day 27 were higher than those values after a single dose (Day 6) and these differences were statistically significant (p≤0.003). The accumulation ratios were greater than one at the 1000 and 2000 mg/kg/day dose levels, indicating that accumulation of ANS 9801-acid occurred after repeated oral administration of ANS 9801 at these dose levels.

The extent (AUC_{24}) of systemic exposure to the metabolite (ANS 9801-acid) was *ca* 22-fold to *ca* 38-fold higher than the exposure to the parent compound (ANS 9801) on Days 6 and 27, indicating that there was extensive metabolism of ANS 9801 to ANS 9801-acid.

In conclusion, the rate and extent of systemic exposure of pregnant female rabbits to ANS 9801 appeared to be characterised by non-linear (dose-dependent) kinetics over the dose range 500 to 2000 mg/kg/day on Day 6 after mating during the oral embryo-foetal toxicity study. Increasing the dose of ANS 9801 above 500 mg/kg/day is likely to result in a disproportionately lower systemic

exposure to ANS 9801 than would be predicted from a linear relationship. After repeated administration (Day 27) there was some evidence that increasing the dose of ANS 9801 resulted in a disproportionately higher systemic exposure to ANS 9801 than would be predicted from a linear relationship, however, the extent of this disproportionality was not great.

The rate and extent of systemic exposure of pregnant female rabbits to ANS 9801-acid, appeared to be characterised by non-linear (dose-dependent) kinetics over the dose range 500 to 2000 mg ANS 9801/kg/day on Day 6 and Day 27 after mating. Increasing the dose of ANS 9801 above 500 mg/kg/day is likely to result in a disproportionately lower systemic exposure to ANS 9801-acid than would be predicted from a linear relationship.

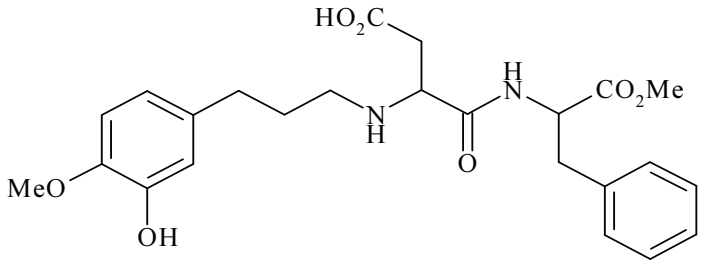
INTRODUCTION

ANS 9801 is currently undergoing development as an artificial sweetener and ANS 9801-acid is a major metabolite. This study (AJO/183) measures the concentrations of ANS 9801 **(I)** and ANS 9801-acid **(II)** in Rabbit plasma samples originating from a toxicity study involving dietary administration of ANS 9801. The internal standards used in the assay are ^{13}C-ANS 9801 **(III)** and ^{13}C-ANS 9801-acid **(IV).**

Concentrations of ANS 9801 and ANS 9801-acid in Rabbit plasma were measured using a liquid chromatographic tandem mass spectrometric (LC-MS/MS) procedure validated at Huntingdon Life Sciences (report AJO/170/014335). ANS 9801 and ANS 9801-acid and their internal standards were extracted from the rabbit plasma samples using solid phase extraction.

Quality control procedures are based upon those outlined at the Washington (1990) conference on 'Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic studies' reported by V P Shah *et al* (1992), J. Pharm. Sci., **81** (3), 309-312, and those reviewed by D. Dadgar *et al* (1995), J. Pharm & Biomed Anal., **13** (2), 89-97. Reanalysis guidelines followed those described by J.R. Lang and S. Bolton (1991), J. Pharm. Biomed. Anal., **9** (5), 357-361.

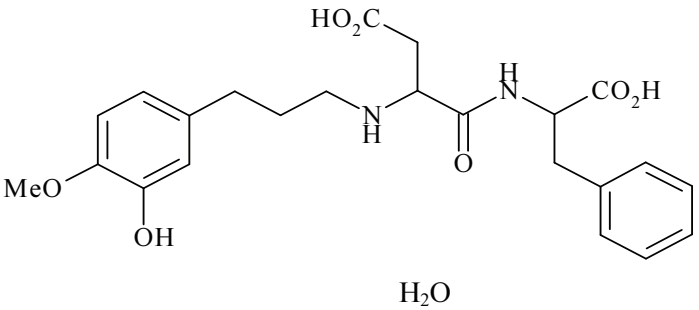
(I) ANS 9801:



H_2O

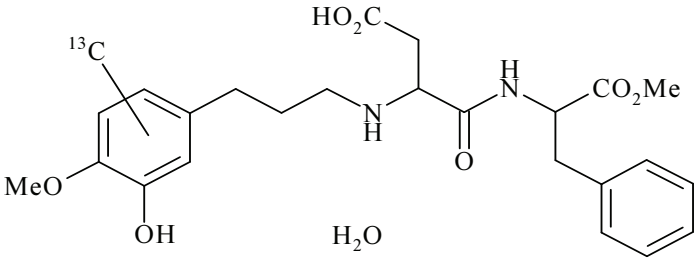
Monoisotopic Mass = 458.2 (free base)
Monoisotopic Mass = 476.2 (monohydrate)
Molecular Formula : $C_{24}H_{30}O_7N_2$ (free base)
Molecular Formula : $C_{24}H_{30}O_7N_2, H_2O$(monohydrate)

(II) ANS 9801-acid:



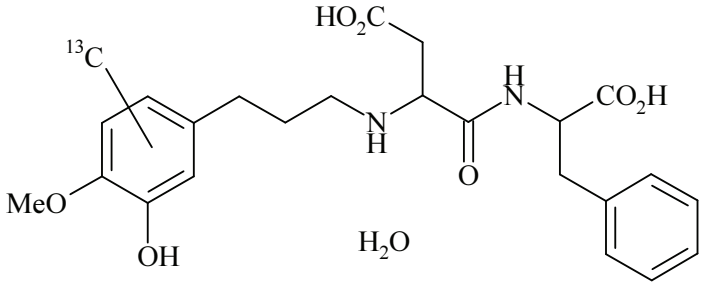
Monoisotopic Mass = 444.2(free base)
Monoisotopic Mass = 462.2 (monohydrate)
Molecular Formula : $C_{23}H_{28}O_7N_2$ (free base)
Monoisotopic Mass : $C_{23}H_{28}O_7N_2$ H_2O (monohydrate)

(III) Internal Standard 1: ^{13}C-ANS 9801



Monoisotopic Mass = 464.2 (free base)
Monoisotopic Mass = 482.2 (monohydrate)
Molecular Formula : $C_{24}H_{30}O_7N_2$ (free base)
Monoisotopic Mass : $C_{24}H_{30}O_7N_2$ H_2O (monohydrate)

(IV) Internal Standard 2: ^{13}C-ANS 9801-acid:



Monoisotopic Mass = 450.2 (free base)
Monoisotopic Mass = 468.2 (monohydrate)
Molecular Formula : $C_{23}H_{28}O_7N_2$ (free base)
Monoisotopic Mass : $C_{23}H_{28}O_7N_2$ H_2O (monohydrate)

STUDY DATES

Experimental (analytical) work phase

Receipt of rabbit plasma samples day 6:	15 August 2001
Receipt of rabbit plasma samples day 27:	06 September 2001
Initiation of experimental phase:	17 August 2001
Completion of experimental phase:	27 September 2001

EXPERIMENTAL PROCEDURE

ORIGIN OF SAMPLES

Female New Zealand White rabbits were dosed with ANS 9801 by oral gavage administration at three different dose levels. Group 1 (control), Group 2 (500 mg/kg/day), Group 3 (1000 mg/kg/day) and Group 4 (2000 mg/kg/day) as described in the protocol. A total of 192 rabbit plasma test samples were received from Toxicology Sciences, HLS. The protocol specified that samples from the control groups were not to be analysed, therefore only 144 samples were analysed. The test samples were received in the Mass Spectrometry department on 15th August and 6th September 2001 for Day 6 and Day 27, respectively. Test samples were stored at *ca.* -80°C and thawed at room temperature prior to analysis. Experimental work was carried out between 17th August and 27th September 2001.

MEASUREMENT OF ANS 9801 AND ANS 9801-ACID IN RABBIT PLASMA

Concentrations of ANS 9801 and ANS 9801-acid in rabbit plasma samples were measured using a liquid chromatographic tandem mass spectrometric method (LC-MS/MS) previously validated at Huntingdon Life Sciences and documented in Report No. AJO/170/014335.

ANS 9801 and ANS 9801-acid and their internal standards (C_{13}-ANS 9801 and C_{13}-ANS 9801-acid) were extracted from rabbit plasma using solid phase extraction. The extracts were chromatographed on a Columbus C_{18} (50 mm × 2 mm ID) column, ionised using a TurboIonSpray™ assisted atmospheric pressure ionisation (API) interface and detected by tandem mass spectrometry (MS/MS) in the multiple reaction monitoring (MRM) mode. The characteristic ion dissociations, under the MS/MS conditions used, of m/z 459 → 102 and m/z 445 → 102 were monitored for ANS 9801 and ANS 9801-acid and the internal standards m/z 465 → 102 and m/z 451 → 102 for ^{13}C-ANS 9801 and ^{13}C-ANS 9801-acid (Appendix 2, Figures 1-8). The assay is robust and sensitive with no significant interference observed from the matrix.

ANALYTICAL EQUIPMENT

Analytical balance:	Mettler AT261	Mettler Instruments, Greifensee, Switzerland.
Autoinjector caps:	11-AC-ST101	Chromacol Ltd., Herts, UK.
Autoinjector caps:	5181-1210	Hichrom Ltd., Reading, Berkshire, UK.
Autoinjector vials:	Polypropylene (300 µl) 11-19-0933	Jones Chromatography, Mid Glamorgan, UK
Autoinjector vials:	Polypropylene (300 µl) 9301-0978	Esslab Ltd., Hadleigh, Essex, UK.
Automatic pipette:	Socorex (10 ml)	Socorex, Lausanne, Switzerland.
Automatic pipettes:	Reference (Various sizes)	Eppendorf, Hamburg, Germany.
Automatic pipette:	Research (20 - 200)	Eppendorf, Hamburg, Germany.
Automatic pipettes:	Microman (Various sizes)	Gilson Medical Electronics, Villiers-le-Bel, France.
Extraction tubes:	5 ml	Sarstedt, Numbrecht, Germany.
Extraction tubes:	3 ml	Sarstedt, Numbrecht, Germany.
Extraction robot:	ASPEC XL4	Gilson Medical Electronics, Villiers-le-Bel, France.
Extraction Cartridges:	ISOLUTE C18 NEC 100mg/1ml	Jones Chromatography Ltd, Mid Glamorgan, UK
Centrifuge:	Mistral 3000i	MSE, Leicester, UK.
HPLC auto-injector:	PE Series 200	PE, Beaconsfield, UK.
HPLC pump:	PE Series 200	PE, Beaconsfield, UK.
HPLC Vac. de-gasser:	PE Series 200	PE, Beaconsfield, UK.
HPLC auto-injector:	Agilent Series 1100	Agilent Technologies, Walbronn, Germany.
HPLC pump:	Agilent Series 1100	Agilent Technologies, Walbronn, Germany.
HPLC Vac. de-gasser:	Agilent Series 1100	Agilent Technologies, Walbronn, Germany.
HPLC column:	Columbus C18 50*2 mm*5µm	Phenomenex, Macclesfield, UK
Mass spectrometer:	PE Sciex API 300 and 365 Series	PE Sciex, Toronto, Canada.
Pasteur pipettes:	Polypropylene	Stratlab, Cambridge, UK.
pH Meter:	Toledo 340	Mettler Instruments, Greifensee, Switzerland.
Repeater pipette:	Multipette 4780	Eppendorf, Hamburg, Germany.
Sample concentrator:	DB-3A	Techne Ltd, Cambridge, UK.

Top Pan Balance:	Scout	Ohaus Corporation, NJ, USA.
Volumetric flasks:	Polypropylene, various sizes	Fisher Scientific, Loughborough UK.
Vortex mixer:	Whirlimixer	Fisher Scientific, Loughborough UK.
UV/Vis detector: [1]	PE 785A	PE, Beaconsfield, UK.
HPLC pump/autoinjector: [1]	Waters 600MS	Waters, Milford, MA, USA
Chart recorder: [1]	Kipp & Zonen BD112	Kipp & Zonen, Holland

[1]Equipment used for solution check only.

MATERIALS

Acetonitrile:	HPLC grade	Fisher Scientific, Loughborough, UK.
ANS 9801:	Analyte	Supplied by Study sponsor *.
	Batch No: 000530	
ANS 9801-acid:	Metabolite	Supplied by Study sponsor *.
	Batch No: AOK-0007	
^{13}C-ANS 9801:	Internal Standard	HRC, Cambs, UK*
	Batch No: LFE ^{13}C-ANS/82	
^{13}C-ANS 9801-acid:	Internal Standard	HRC, Cambs, UK*
	Batch No: LFE ^{13}C-ACID/83	
Control matrix:	Rabbit plasma	Harlan Ltd, Loughborough, UK.
Citric acid:	>99.5% anhydrous	Sigma-Aldrich, Dorset, UK.
Diethyl-4 Nitrophenyl phosphate (Paraoxon):	TECH., 90%	Sigma-Aldrich, Dorset, UK.
Ethanol:	Absolute Grade	Fisher Scientific, Loughborough, UK.
Formic acid:	Analytical Reagent	Fisher Scientific, Loughborough, UK.
Hydrochloric acid:	SG 1.16, (32%)	Fisher Scientific, Loughborough, UK
Methanol:	HPLC grade	Fisher Scientific, Loughborough, UK.
Nitrogen:	Liquid (99.998%)	BOC Ltd, London , UK
Water:	Maxima HPLC (18.2 MΩ)	Elga LTD., High Wycombe, UK.

* Certificates of analysis presented in Appendix 4.

PREPARATION OF REAGENTS

0.5 M HCl

50 ml of HCl was diluted to 1000 ml with deionised water.

Formic acid 0.1%

Formic acid (1 ml) was diluted to 1000 ml with deionised water.

Mobile phase/reconstitution solvent/needle rinse: (25/75) acetonitrile/formic acid 0.1%

125 ml of acetonitrile was added to 375 ml of formic acid 0.1 %.

Formic acid 1% (ASPEC RESERVOIR)

Formic acid (10 ml) was diluted to 1000 ml with deionised water.

1M Citric acid

19.24 g of citric acid was diluted to 100 ml with deionised water.

Paraoxon (5mg/ml)

19.6 µl of paraoxon was added to a 5 ml volumetric flask and made up to volume with ethanol.

Density of Paraoxon: 1.274 mg/µl.

Preparation of stabilised rabbit plasma

To 20 ml of rabbit plasma, paraoxon (5mg/ml, 120 µl) and citric acid (1M, 2 ml) were added.

PREPARATION OF STOCK SOLUTIONS

ANS 9801

Two stock solutions of analyte (Calibration Stock and QC Stock) were prepared in an identical manner. Approximately 2.5 mg of analyte was accurately weighed and dissolved in 25 ml of methanol, giving a 100µg/ml stock solution. The stock solutions were checked against each other using LC-UV.

As the purity of the compound is 100 % and supplied in a monohydrate form, 2.5 mg of the pure compound is equivalent to:

Target weighing × (100/Purity) × (RMM of analyte salt/(RMM of free analyte × no of analyte molecules in salt))

2.5 × (100/100) × (476.52676/(458.51148× 1)) =2.60 mg

ANS 9801-acid

Two stock solutions of metabolite (Calibration Stock and QC Stock) were prepared in an identical manner. Approximately 2.5 mg of metabolite were accurately weighed and dissolved in 25 ml of methanol, giving a 100µg/ml stock solution. The Stock solutions were checked against each other using LC-UV.

As the purity of the compound is 100% and supplied in a monohydrate form, 2.5 mg of the pure compound is equivalent to:

Target weighing × (100/Purity) × (RMM of analyte salt/(RMM of free analyte × no of analyte molecules in salt))

2.5 × (100/100) × (462.49988/(444.4846×1)) = 2.60 mg

PREPARATION OF INTERNAL STANDARD STOCK SOLUTION

Approximately 2.5 mg of internal standard ^{13}C-ANS 9801 was accurately weighed and dissolved in 25 ml of methanol, giving a 100 µg/ml stock solution (IS Stock).

As the purity of the compound is 100% and supplied in a monohydrate form, 2.5 mg of the pure compound is equivalent to:

Target weighing × (100/Purity) × (RMM of analyte salt/(RMM of free analyte × no of analyte molecules in salt))

2.5 × (100/100) × (482.48089/464.46561 × 1)= 2.60mg

PREPARATION OF INTERNAL STANDARD STOCK SOLUTION

Approximately 2.5 mg of internal standard ^{13}C-ANS 9801-acid was accurately weighed and dissolved in 25 ml of methanol, giving a 100 µg/ml stock solution (IS Stock).

As the purity of the compound is 100% and supplied in a monohydrate form, 2.5 mg of the pure compound is equivalent to:

Target weighing × (100/Purity) × (RMM of analyte salt/(RMM of free analyte × no of analyte molecules in salt))

2.5 × (100/100) × (468.45401/(450.43873×1)) = 2.60 mg

All Stock solutions were stored in the dark at 4°C when not in use.

PREPARATION OF INTERNAL STANDARD WORKING SOLUTION (100 ng/ml)

Each IS Stock solution (100 µl) were diluted to 100 ml with methanol in a volumetric flask.

PREPARATION OF INTERMEDIATE SOLUTIONS

INTERMEDIATE SOLUTION A (10 μg/ml)

2500 μl of ANS 9801 and ANS 9801-acid Stock solutions (100 μg/ml) were diluted to 25 ml with methanol.

INTERMEDIATE SOLUTION B (1 μg/ml)

250 μl of ANS 9801 and ANS 9801-acid Stock solutions (100 μg/ml) were diluted to 25 ml with methanol.

PREPARATION OF CALIBRATION STANDARD WORKING SOLUTIONS

SOLUTION A (200 ng/ml)

500 μl of Intermediate solution A (10 μg/ml) were diluted to 25 ml with methanol.

SOLUTION B (100 ng/ml)

250 μl of Intermediate solution A (10 μg/ml) were diluted to 25 ml with methanol.

SOLUTION C (40 ng/ml)

100 μl of Intermediate solution A (10 μg/ml) were diluted to 25 ml with methanol.

SOLUTION D (20 ng/ml)

50 μl of the Intermediate solution A (10 μg/ml) were diluted to 25 ml with methanol.

SOLUTION E (10 ng/ml)

250 μl of the Intermediate solution B (1 μg/ml) were diluted to 25 ml with methanol.

SOLUTION F (4 ng/ml)

100μl of the Intermediate solution B (1 μg/ml) were diluted to 25 ml with methanol.

SOLUTION G (2 ng/ml)

500 μl of Calibration solution B (100 ng/ml) were diluted to 25 ml with methanol.

SOLUTION H (1 ng/ml)

250 μl of Calibration solution B (100 ng/ml) were diluted to 25 ml with methanol.

PREPARATION OF SST SOLUTION (1.5 ng/ml of ANS 9801 and ANS 9801-acid)

Calibration solution A (750 µl) and each IS Stock solution for C_{13}-ANS 9801 and C_{13}-ANS 9801-acid (50 µl) were diluted to 100 ml with mobile phase.

PREPARATION OF QUALITY CONTROL STANDARD WORKING SOLUTIONS

QC SOLUTION A (10 µg/ml)

2500 µl of each of the QC stock solutions (100 µg/ml) of ANS 9801 and ANS 9801 acid were diluted to 25 ml with methanol in a volumetric flask.

QC SOLUTION B (1 µg/ml)

250 µl of each of the QC stock solutions (100 µg/ml) of ANS 9801 and ANS 9801-acid were diluted to 25 ml with methanol in a volumetric flask.

QC SOLUTION C (20 ng/ml)

50 µl of QC solution A were diluted to 25 ml of methanol in a volumetric flask.

All solutions were stored at 4°C in the dark when not in use.

PREPARATION OF BULK QC'S

QC LOW (1.5 ng/ml)

375 µl of QC solution C (20 ng/ml) were made up to 5ml with 4625ml stabilised rabbit plasma using pipettes.

QC MEDIUM (7 ng/ml)

35 µl of QC solution B (1 µg/ml) were made up to 5ml with 4965ml stabilised rabbit plasma using pipettes.

QC HIGH (70 ng/ml)

350 µl of QC solution B (1 µg/ml) were made up to 5ml with 4650ml stabilised rabbit plasma using pipettes.

100 µl aliquots of each bulk QC was stored at -80°C until required for analysis. Prior to analysis, 50 µl of internal standard solution and 50 µl of methanol was added to each QC aliquot.

PREPARATION OF CALIBRATION STANDARDS

To stabilised control rabbit plasma aliquots (100 µl), 50 µl of internal standard working solution (except blank) was added, followed by an appropriate volume of analyte standard solution (as listed in the following table). Any difference between spiked volumes was adjusted with methanol.

Matrix concentration (ng/ml)	**Vol. of standard solution (µl) spiked into matrix**	**Vol. of MeOH (µl) added to matrix**	**Standard solution**
BLANK	0	100	n/a
Zero	0	50	n/a
0.5	50	0	H
1	50	0	G
2	50	0	F
5	50	0	E
10	50	0	D
20	50	0	C
50	50	0	B
100	50	0	A

SAMPLE EXTRACTION PROCEDURE

Calibration standards

To nine stabilised control rabbit plasma aliquots (100 µl), 50 µl of internal standard working solution was added, followed by 50 µl of ANS 9801 and ANS 9801-acid calibration standard or methanol for the zero standard.

Plasma blank

To Control rabbit plasma aliquot (100 µl), 100 µl of methanol was added.

Test sample and QC preparation

Aliquots (100 µl) of samples were transferred to test tubes. 50 µl of working internal standard solution (100 ng/ml) and 50 µl of methanol were added.

Procedure

1. Hydrochloric Acid (HCl) (500 µl, 0.5 M) was added to all the samples and the solutions were vortex mixed (*ca* 5 sec) and centrifuged (3500 r.p.m. for 10 minutes).

2. The samples were loaded onto ISOLUTE C18 100mg/1ml cartridges preconditioned with methanol (1.6 ml) and HCl 0.5M (1.6 ml).

3. Washed with 0.5M HCl (0.8ml).

4. Eluted with acetonitrile (0.8ml).

5. Evaporated to dryness at approximately 40°C under a gentle stream of nitrogen.

6. The samples were reconstituted in mobile phase (100 µl) by vortex mixing for *ca.* 5 seconds. The solutions were transferred into polypropylene auto-sampler vials centrifuged (3500 r.p.m. for 10 minutes) and an aliquot of 5-25 µl was analysed by LC-MS/MS.

A schematic representation of this procedure is shown in Appendix 3, Figure 1.

LC-MS/MS

Column:	Columbus C_{18} 50 mm × 2 mm × 5 μm (Phenomenex)
Injection volume:	5 μl – 25 μl
Pre-injection flushes:	2 at 250 μl
Post-injection flushes:	2 at 250 μl
Column Temperature:	Ambient
Mobile phase:	(25:75) acetonitrile: aqueous formic acid 0.1%
Mobile phase flow rate:	0.2 ml/min

Mass spectrometry

Ionisation:	Atmospheric pressure ionisation (API) using a TurboIonSpray™ source in the positive ion mode
Collision gas:	Nitrogen
Collision energy:	*ca* 30 eV
Dwell Time:	250 ms for each MRM transition
Pause Time:	50 ms
Ions monitored:	m/z 459→ 102 for analyte ANS9801 m/z 445 → 102 for metabolite ANS9801-acid m/z 465 →102 for IS ^{13}C-ANS 9801 m/z 451 →102 for IS ^{13}C-ANS 9801-acid

ACCEPTABILITY OF ANALYTICAL BATCHES

The acceptability of each batch of test samples depended on the data from the calibration standards and the QC samples fulfilling the following requirements:

A minimum of four out of six QC's being within ± 15% (± 20% at QC low concentration) of their respective nominal concentrations. (No more than one QC may be greater than ± 15% or ± 20% at QC low at any one concentration).

Only data from accepted batches are reported.

DATA PROCESSING

Chromatographic

All data collection, processing (peak area integration and quantification) and storage was performed using MACQUAN software (V1.5, PE SCIEX) associated with the mass spectrometer.

The mass spectrometer response (peak area ratios of analyte vs. internal standard of each calibration standards) were plotted against the theoretical (prepared) concentrations, and subjected to least squares regression analysis to provide values for slope, the y intercept, correlation coefficient and back-calculated concentrations.

The results for the summary of the calibration line data, calibration standards, quality control samples and typical calibration line and chromatograms are presented in Appendix 3, Tables 1-6 and Figures 2-7.

Toxicokinetic and statistical analysis

Maximum mean plasma concentrations (C_{max}), their times of occurrence (T_{max}) and the mean plasma concentrations at 24 hours post-dose (C_{24}) were the observed values. Areas under the mean plasma concentration-time curves within a 24 hour dosing interval (AUC_{24}), and their variances, were estimated by the linear trapezoidal rule according to Bailer (1988; see statistics report, Appendix 1). C_{max} and AUC_{24} values, taken as indices of systemic exposure, were adjusted for dose and comparisons made between dose levels (test for non-proportionality) and between days of sampling. Full details of the statistical analysis are presented in Appendix 1.

AUC_{24} values were calculated using the statistical package SAS 6.12.

LOCATION OF ORIGINAL DATA

This report has been compiled from original data generated by the Department of Mass Spectrometry, (Notebook 2001/0694) and the Department of Pharmacokinetics (Notebook PKN 01/402) of Huntingdon Life Sciences Ltd. All the original data and a copy of this report will be stored in the archives of Huntingdon Life Sciences Ltd. for a minimum of five years from the date of issue of this report.

After the five year retention period the Sponsor will be contacted and their advice sought on the return, disposal or further retention of the materials.

RESULTS AND DISCUSSION

LC-MS/MS

The reversed phase column, with a mobile phase flow rate of 0.2 ml/min, gave a retention time for ANS 9801 and ANS 9801-acid and their internal standards of between 1.5 and 3 minutes.

The API TurboIonSpray™ LC-MS/MS interface gave optimum results in the positive ion mode. ANS 9801 and ANS 9801-acid and their internal standards produced quasi-molecular ions at m/z 459, 445, 465 and 451, respectively. Fragmentation of these ions (Appendix 2, Figures 1-8) using CAD (collision activated dissociation) with a collision energy of *ca.* 30 eV in the Q2 region of the mass spectrometer resulted in strong product ions predominantly at m/z 102 for all compounds. Monitoring the product ions from the quasi-molecular ions using MRM (multiple reaction monitoring) gave excellent specificity and sensitivity.

CALIBRATION

The assay was linear over the range 0.5-100 ng/ml. Weighted least square regression (1/y) was used for all assay batches. Calibration data for all assay batches are summarised in Appendix 3, Table 1 and 2 for ANS 9801 and ANS 9801-acid respectively. Values for the slope and correlation coefficient were repeatable and a negligible intercept was observed. The mean slope for ANS 9801 was 0.0356 with an inter-batch coefficient of variation (CV) of 15.3%. The mean slope for ANS 9801-acid was 0.0259 with an inter-batch coefficient of variation (CV) of 11.7%. Representative calibration lines are shown in Appendix 3, Figures 2 and 3.

Back-calculated concentrations of calibration standards are presented in Appendix 3, Table 3 and 4 for ANS 9801 and ANS 9801-acid respectively. For ANS 9801, inter-batch accuracy (relative error) was 3.22% at 0.5 ng/ml and within or equals to ±4.42% at all other concentrations. Inter-batch precision (CV) was 8.27% at 0.5 ng/ml and less than or equals to 5.71% at all other concentrations. For ANS 9801-acid, inter-batch accuracy (relative error) was 8.07% at 0.5 ng/ml and within or equal to ±5.93% at all other concentrations. Inter-batch precision (CV) was 10.2% at 0.5 ng/ml and less than or equals to 8.28% at all other concentrations.

QUALITY CONTROL

Results for the low, medium and high QC samples are presented in Appendix 3, Table 5 and 6 for ANS 9801 and ANS 9801-acid respectively. For ANS 9801, inter-batch accuracy (relative error) was 3.82% at the low QC level and within or equal to ±4.56% at all other concentrations. Inter-batch precision (CV) was 6.92% at the low QC level and less than or equals to 4.16% at all other concentrations. For ANS 9801-acid, inter-batch accuracy (relative error) was -4.38% at the low QC level and within or equals to ±2.86% at all other concentrations. Inter-batch precision (CV) was 8.94% at the low QC level and less than or equals to 7.66% at all other concentrations.

ANALYSIS OF TEST SAMPLES

The partial validation in rabbit plasma was ongoing at the time of analysis of the samples from AJO/183, therefore not all stability experiments had been performed. When the day 6 samples were analysed they had been kept for longer than 1 week, which, at the time, was the actual verified stability period. However, at the time of reporting the data from this study, ANS 9801 and ANS 9801-acid were shown to be stable for 3 months, which covers the storage period of all samples analysed.

Blood samples were taken on Day 6 and Day 27 of pregnancy (Days 1 and 22 of treatment) during an embryo-foetal toxicity study, in order to assess the systemic exposure of pregnant female rabbits to ANS 9801 and its metabolite ANS 9801-acid, following daily oral gavage administration of ANS 9801 at dose levels of 500, 1000 and 2000 mg/kg/day from Day 6 to Day 27 after mating. Plasma concentrations of ANS 9801 and ANS 9801-acid (both measured as anhydrous bases) in samples taken up to 24 hours post-dose were measured by a validated liquid chromatographic - tandem mass spectrometric (LC-MS/MS) method and are presented in Tables 1 to 4. The mean plasma concentration-time profiles are illustrated in Figures 1 to 4.

ANS 9801

Maximum mean plasma concentrations (C_{max}) of ANS 9801, their times of occurrence (T_{max}), mean plasma ANS 9801 concentrations at 24 hours post-dose (C_{24}) and the areas under the mean plasma ANS 9801 concentration-time curves estimated up to 24 hours post-dose (AUC_{24}) on Day 6 and Day 27 after mating are presented in Table 5, and the C_{max} and AUC_{24} values are summarised below:

Dose level	C_{max} (ng/ml)		AUC_{24} (ng.h/ml)	
(mg/kg/day)	Day 6	Day 27	Day 6	Day 27
500	944	847	4982	3906
1000	714	1178	3737	11594
2000	1070	2980	8382	22367

The times at which the maximum mean plasma concentrations occurred (T_{max}) were generally 0.5 hours post-dose, and in the range 0.5 to 1 hour, indicating that absorption was rapid.

The mean plasma concentrations of ANS 9801, at 24 hours post-dose (C_{24}), were quantifiable in all dose groups on Day 6 and Day 27 after mating, therefore, animals were continuously exposed to quantifiable concentrations of ANS 9801 during a dosing interval.

The relationships between maximum mean plasma concentrations (C_{max}) of ANS 9801, areas under the mean plasma ANS 9801 concentration-time curves (AUC_{24}) and dose level are presented below:

Dose level	Dose level	C_{max} ratio		AUC_{24} ratio	
(mg/kg/day)	ratio	Day 6	Day 27	Day 6	Day 27
500	1	1	1	1	1
1000	2.0	0.8	1.4	0.8	3.0
2000	4.0	1.1	3.5	1.7	5.7

The rate of systemic exposure of pregnant female rabbits to ANS 9801, characterised by C_{max}, was similar at each dose level on Day 6 after mating (Figure 5). On Day 27, the C_{max} values increased with increasing dose over the dose range 500 to 2000 mg/kg/day (Figure 5), however, these increases were less than the proportionate dose increment. Overall, there was statistically significant evidence of non-proportionality (p=0.025; Appendix 1).

The extent of systemic exposure of pregnant female rabbits to ANS 9801, characterised by AUC_{24}, was similar at the 500 and 1000 mg/kg/day dose levels on Day 6 after mating, but increased at the 2000 mg/kg/day dose level (Figure 6). However, this increase was *ca* 58% lower than the proportionate dose increment (compared with the value at the 500 mg/kg/day dose level), and there was statistically significant evidence of non-proportionality (p<0.001; Appendix 1). On Day 27, the AUC_{24} values increased with increasing dose over the dose range 500 to 2000 mg/kg/day (Figure 6). These increases were greater than the proportionate dose increment, and there was statistically significant evidence of non-proportionality (p=0.002; Appendix 1). At the highest dose level (2000 mg/kg/day) the AUC_{24} values on Day 27 were *ca* 1.4-fold higher, than those values predicted from a linear relationship.

After repeated oral doses (Day 27) the rate (C_{max}) of systemic exposure of pregnant female rabbits to ANS 9801 was similar to that after a single dose (Day 6) at the lowest dose level (500 mg/kg/day; Figure 7). At the two higher dose levels (1000 and 2000 mg/kg/day), however, the C_{max} values were higher after repeated oral doses than those values after a single dose (Figure 7) and overall these differences were statistically significant (p=0.031; Appendix 1). The extent (AUC_{24}) of systemic exposure of pregnant female rabbits to ANS 9801 at the lowest dose level (500 mg/kg/day) was lower than that after a single dose (Day 6; Figure 7) and this difference was statistically significant (p=0.034; Appendix 1). At the two higher dose levels (1000 and 2000 mg/kg/day) the AUC_{24} values were higher after repeated oral doses than those values after a single dose (Day 6), and these differences were statistically significant (p<0.001; Appendix 1). The accumulation ratios, based on AUC_{24} values, are presented below:

Dose level (mg/kg/day)	Accumulation ratio
500	0.8
1000	3.1
2000	2.7

The accumulation ratios were greater than one at the 1000 and 2000 mg/kg/day dose levels, indicating that accumulation occurred after repeated oral administration of ANS 9801 at these dose levels.

ANS 9801-acid

Maximum mean plasma concentrations (C_{max}) of ANS 9801-acid, their times of occurrence (T_{max}), mean plasma ANS 9801-acid concentrations at 24 hours post-dose (C_{24}) and the areas under the mean plasma ANS 9801-acid concentration-time curves estimated up to 24 hours post-dose (AUC_{24}) on Day 6 and Day 27 after mating are presented in Table 6, and the C_{max} and AUC_{24} values are summarised below:

Dose level	C_{max} (ng/ml)		AUC_{24} (ng.h/ml)	
(mg/kg/day)	Day 6	Day 27	Day 6	Day 27
500	10513	14338	151896	147414
1000	13694	21382	117112	268999
2000	18923	30400	274417	486206

The times at which the maximum mean plasma concentrations occurred (T_{max}) were generally 1 hour post-dose, and in the range 0.5 to 1 hour, indicating that metabolism from ANS 9801 to its major metabolite ANS 9801-acid, was rapid.

The mean plasma concentrations of ANS 9801-acid at 24 hours post-dose (C_{24}) were quantifiable in all dose groups on Day 6 and Day 27 after mating, therefore, animals were continuously exposed to quantifiable concentrations of ANS 9801-acid during a dosing interval.

The relationships between maximum mean plasma concentrations (C_{max}) of ANS 9801-acid, areas under the mean plasma ANS 9801-acid concentration-time curves (AUC_{24}) and dose level are presented below:

Dose level	Dose level	C_{max} ratio		AUC_{24} ratio	
(mg/kg/day)	ratio	Day 6	Day 27	Day 6	Day 27
500	1	1	1	1	1
1000	2.0	1.3	1.5	0.8	1.8
2000	4.0	1.8	2.1	1.8	3.3

The rate and extent of systemic exposure of pregnant female rabbits to ANS 9801-acid, characterised by C_{max} and AUC_{24} respectively, generally increased with increasing dose over the dose range 500 to 2000 mg/kg/day on Day 6 and Day 27 after mating. These increases were less than the proportionate dose increment (Figures 8 and 9), and there was statistically significant evidence of non-proportionality ($p \leq 0.030$; Appendix 1) with the exception of the AUC_{24} values on Day 27 which did not reach statistical significance ($p=0.31$; Appendix 1). Overall, the C_{max} and AUC_{24} values at the highest dose level (2000 mg/kg/day) were *ca* 51% and *ca* 36% lower, respectively, than those values predicted from a linear relationship.

After repeated oral doses of ANS 9801 (Day 27) the rate (C_{max}) of systemic exposure of pregnant female rabbits to ANS 9801-acid was *ca* 1.4 to 1.6-fold higher than those values after a single dose (Figure 10), and these differences were statistically significant ($p=0.038$; Appendix 1). The extent (AUC_{24}) of systemic exposure to ANS 9801-acid at the lowest dose level (500 mg/kg/day) on Day 27 after mating was similar to that after a single dose (Day 6; Figure 10), and there was no statistically significant evidence for a time (day of sampling) related difference ($p=0.78$; Appendix 1). However, at the two higher dose levels (1000 and 2000 mg/kg/day) the AUC_{24} values on Day 27 were higher than those values after a single dose (Day 6; Figure 10) and these differences were statistically significant ($p \leq 0.003$; Appendix 1). The accumulation ratios, based on AUC_{24} values, are presented below:

Dose level (mg/kg/day)	Accumulation ratio
500	1.0
1000	2.3
2000	1.8

The accumulation ratios were greater than one at the 1000 and 2000 mg/kg/day dose levels, indicating that accumulation of ANS 9801-acid occurred after repeated oral administration of ANS 9801 at these dose levels.

The extent (AUC_{24}) of systemic exposure to the metabolite (ANS 9801-acid) was *ca* 22-fold to *ca* 38-fold higher than the exposure to the parent compound (ANS 9801) on Days 6 and 27, indicating that there was extensive metabolism of ANS 9801 to ANS 9801-acid. The metabolite ratios, based on AUC_{24} values, are presented below:

Dose level	Metabolite ratios	
(mg/kg/day)	Day 6	Day 27
500	30.5	37.7
1000	31.3	23.2
2000	32.7	21.7

The metabolite ratios at the 1000 and 2000 mg/kg/day dose levels on Day 27 were lower than those on Day 6.

In conclusion, the rate and extent of systemic exposure of pregnant female rabbits to ANS 9801 appeared to be characterised by non-linear (dose-dependent) kinetics over the dose range 500 to 2000 mg/kg/day on Day 6 after mating during the oral embryo-foetal toxicity study. Increasing the dose of ANS 9801 above 500 mg/kg/day is likely to result in a disproportionately lower systemic exposure to ANS 9801 than would be predicted from a linear relationship, which is consistent with the possibility of dissolution-rate limited absorption. After repeated administration (Day 27) there was some evidence that increasing the dose of ANS 9801 resulted in a disproportionately higher systemic exposure to ANS 9801 than would be predicted from a linear relationship, however, the extent of this disproportionality was not great.

The rate and extent of systemic exposure of pregnant female rabbits to ANS 9801-acid, appeared to be characterised by non-linear (dose-dependent) kinetics over the dose range 500 to 2000 mg ANS 9801/kg/day on Day 6 and Day 27 after mating. Increasing the dose of ANS 9801 above 500 mg/kg/day is likely to result in a disproportionately lower systemic exposure to ANS 9801-acid than would be predicted from a linear relationship.

TABLE 1

Plasma concentrations of ANS 9801 on Day 6 after mating (Day 1 of treatment) following daily oral gavage administration of ANS 9801 to pregnant female rabbits

500 mg/kg/day

Time (hours)	Concentration (ng/ml)							
	Rabbit numbers in parentheses						Mean	sd
Predose	BLQ	(7)	BLQ	(8)	BLQ	(9)	-	-
0.5	396[a]	(10)	577	(11)	1311	(12)	944	-
1	913	(7)	1044	(8)	677	(9)	878	186
2	260[a]	(10)	122	(11)	455	(12)	288	-
4	238	(7)	217	(8)	162	(9)	206	39
8	311[a]	(10)	188	(11)	143	(12)	166	-
12	231	(7)	193	(8)	175	(9)	200	29
24	169[a]	(10)	103	(11)	77.6	(12)	90.1	-

1000 mg/kg/day

Time (hours)	Concentration (ng/ml)							
	Rabbit numbers in parentheses						Mean	sd
Predose	BLQ	(13)	BLQ	(14)	BLQ	(15)	-	-
0.5	646	(16)	925	(17)	572	(18)	714	186
1	694	(13)	620	(14)	542	(15)	619	76
2	251	(16)	643	(17)	187	(18)	360	247
4	90.4	(13)	139	(14)	52.3	(15)	93.9	43.4
8	101	(16)	97.1	(17)	87.2	(18)	95.0	7.1
12	115	(13)	124	(14)	101	(15)	113	11
24	241	(16)	80.3	(17)	81.7	(18)	134	92

2000 mg/kg/day

Time (hours)	Concentration (ng/ml)							
	Rabbit numbers in parentheses						Mean	sd
Predose	BLQ	(19)	BLQ	(20)	BLQ	(21)	-	-
0.5	887	(22)	648[a]	(23)	1557[a]	(24)	887[b]	-
1	1127	(19)	1302	(20)	781	(21)	1070	265
2	722	(22)	688[a]	(23)	1427[a]	(24)	722[b]	-
4	78.4	(19)	511	(20)	820	(21)	470	373
8	259	(22)	116[a]	(23)	2231[a]	(24)	259[b]	-
12	117	(19)	203	(20)	765	(21)	362	352
24	118	(22)	88.1[a]	(23)	1031[a]	(24)	118[b]	-

BLQ Below the limit of quantification (<0.5 ng/ml)

[a] Animal not pregnant, excluded from calculation of mean

[b] Mean based on one value

TABLE 2

Plasma concentrations of ANS 9801 on Day 27 after mating (Day 22 of treatment) following daily oral gavage administration of ANS 9801 to pregnant female rabbits

500 mg/kg/day

Time (hours)	Concentration (ng/ml) Rabbit numbers in parentheses						Mean	sd
Predose	42.2	(7)	88.7	(8)	67.3	(9)	66.1	23.3
0.5	NS	(10)	777	(11)	917	(12)	847	-
1	604	(7)	593	(8)	365	(9)	521	135
2	NS	(10)	235	(11)	200	(12)	218	-
4	218	(7)	290	(8)	268	(9)	259	37
8	NS	(10)	142	(11)	107	(12)	125	-
12	102	(7)	214	(8)	145	(9)	154	56
24	NS	(10)	8.61	(11)	73.2	(12)	40.9	-

1000 mg/kg/day

Time (hours)	Concentration (ng/ml) Rabbit numbers in parentheses						Mean	sd
Predose	491	(13)	134	(14)	31.0	(15)	219	241
0.5	1111	(16)	776	(17)	318	(18)	735	398
1	906	(13)	684	(14)	1944	(15)	1178	673
2	889	(16)	1778	(17)	317	(18)	995	736
4	882	(13)	365	(14)	221	(15)	490	348
8	360	(16)	286	(17)	250	(18)	299	56
12	569	(13)	203	(14)	240	(15)	337	202
24	195	(16)	1172	(17)	351	(18)	573	525

2000 mg/kg/day

Time (hours)	Concentration (ng/ml) Rabbit numbers in parentheses						Mean	sd
Predose	560	(19)	211	(20)	576	(21)	449	206
0.5	2980	(22)	1827[a]	(23)	NS	(24)	2980[b]	-
1	2136	(19)	2029	(20)	2477	(21)	2214	234
2	1840	(22)	837[a]	(23)	NS	(24)	1840[b]	-
4	820	(19)	544	(20)	760	(21)	708	145
8	891	(22)	411[a]	(23)	NS	(24)	891[b]	-
12	872	(19)	325	(20)	734	(21)	644	285
24	918	(22)	314[a]	(23)	NS	(24)	918[b]	-

BLQ Below the limit of quantification (<0.5 ng/ml)
NS no sample
[a] Animal not pregnant, excluded from calculation of mean
[b] Mean based on one value

TABLE 3

Plasma concentrations of ANS 9801-acid on Day 6 after mating (Day 1 of treatment) following daily oral gavage administration of ANS 9801 to pregnant female rabbits

500 mg/kg/day

Time (hours)	Concentration (ng/ml)							
	Rabbit numbers in parentheses						Mean	sd
Predose	BLQ	(7)	BLQ	(8)	BLQ	(9)	-	-
0.5	6031[a]	(10)	6783	(11)	14242	(12)	10513	-
1	11816	(7)	9094	(8)	8529	(9)	9813	1758
2	6840[a]	(10)	9784	(11)	7572	(12)	8678	-
4	8014	(7)	10034	(8)	6517	(9)	8188	1765
8	7605[a]	(10)	5358	(11)	3571	(12)	4464	-
12	8952	(7)	7633	(8)	7100	(9)	7895	953
24	4722[a]	(10)	4012	(11)	2882	(12)	3447	-

1000 mg/kg/day

Time (hours)	Concentration (ng/ml)							
	Rabbit numbers in parentheses						Mean	sd
Predose	BLQ	(13)	BLQ	(14)	BLQ	(15)	-	-
0.5	5000	(16)	8067	(17)	10004	(18)	7690	2523
1	21129	(13)	11873	(14)	8080	(15)	13694	6712
2	10033	(16)	6482	(17)	6023	(18)	7513	2195
4	6888	(13)	7562	(14)	5628	(15)	6693	982
8	4138	(16)	2644	(17)	1537	(18)	2773	1306
12	4079	(13)	3995	(14)	5333	(15)	4469	749
24	6950	(16)	3079	(17)	2374	(18)	4134	2464

2000 mg/kg/day

Time (hours)	Concentration (ng/ml)							
	Rabbit numbers in parentheses						Mean	sd
Predose	BLQ	(19)	BLQ	(20)	BLQ	(21)	-	-
0.5	9516	(22)	5837[a]	(23)	19435[a]	(24)	9516[b]	-
1	16883	(19)	25773	(20)	14112	(21)	18923	6092
2	18891	(22)	11354[a]	(23)	30979[a]	(24)	18891[b]	-
4	9472	(19)	12850	(20)	19721	(21)	14014	5223
8	11884	(22)	4792[a]	(23)	45318[a]	(24)	11884[b]	-
12	9007	(19)	8729	(20)	18785	(21)	12174	5727
24	6693	(22)	4011[a]	(23)	22834[a]	(24)	6693[b]	-

BLQ Below the limit of quantification (<0.5 ng/ml)

[a] Animal not pregnant, excluded from calculation of mean

[b] Mean based on one value

TABLE 4

Plasma concentrations of ANS 9801-acid on Day 27 after mating (Day 22 of treatment) following daily oral gavage administration of ANS 9801 to pregnant female rabbits

500 mg/kg/day

Time (hours)	Concentration (ng/ml)							
	Rabbit numbers in parentheses						Mean	sd
Predose	3771	(7)	5963	(8)	6768	(9)	5501	1551
0.5	NS	(10)	10623	(11)	13534	(12)	12078	-
1	16213	(7)	12618	(8)	14182	(9)	14338	1802
2	NS	(10)	6877	(11)	6459	(12)	6668	-
4	9826	(7)	15107	(8)	10457	(9)	11797	2884
8	NS	(10)	5586	(11)	4019	(12)	4802	-
12	4237	(7)	9325	(8)	4872	(9)	6145	2773
24	NS	(10)	2505	(11)	2658	(12)	2581	-

1000 mg/kg/day

Time (hours)	Concentration (ng/ml)							
	Rabbit numbers in parentheses						Mean	sd
Predose	16367	(13)	3829	(14)	1726	(15)	7307	7916
0.5	19968	(16)	12978	(17)	8412	(18)	13786	5821
1	29993	(13)	10091	(14)	24061	(15)	21382	10218
2	25480	(16)	18472	(17)	7532	(18)	17161	9045
4	19492	(13)	9685	(14)	6443	(15)	11873	6794
8	12131	(16)	8677	(17)	7655	(18)	9488	2346
12	23502	(13)	5306	(14)	6741	(15)	11850	10117
24	7567	(16)	8605	(17)	8894	(18)	8356	698

2000 mg/kg/day

Time (hours)	Concentration (ng/ml)							
	Rabbit numbers in parentheses						Mean	sd
Predose	21049	(19)	5463	(20)	17853	(21)	14788	8232
0.5	26145	(22)	16871[a]	(23)	NS	(24)	26145[b]	-
1	33484	(19)	25921	(20)	31795	(21)	30400	3970
2	19500	(22)	19399[a]	(23)	NS	(24)	19500[b]	-
4	24082	(19)	13257	(20)	19536	(21)	18958	5436
8	26235	(22)	9629[a]	(23)	NS	(24)	26235[b]	-
12	22814	(19)	7404	(20)	19356	(21)	16525	8086
24	20563	(22)	9030[a]	(23)	NS	(24)	20563[b]	-

BLQ Below the limit of quantification (<0.5 ng/ml)
NS no sample
[a] Animal not pregnant, excluded from calculation of mean
[b] Mean based on one value

TABLE 5

Toxicokinetic parameters of ANS 9801 on Day 6 and on Day 27 after mating (Day 1 and 22 of treatment) following daily oral administration of ANS 9801 to pregnant female rabbits

Day 6

Dose level (mg/kg/day)	C_{max} (ng/ml)	T_{max} (hours)	C_{24} (ng/ml)	AUC_{24} (ng.h/ml)
500	944	0.5	90.1	4982
1000	714	0.5	134	3737
2000	1070	1	118	8382

Day 27

Dose level (mg/kg/day)	C_{max} (ng/ml)	T_{max} (hours)	C_{24} (ng/ml)	AUC_{24} (ng.h/ml)
500	847	0.5	40.9	3906
1000	1178	1	572	11594
2000	2980	0.5	917	22367

TABLE 6

Toxicokinetic parameters of ANS 9801-acid on Day 6 and on Day 27 after mating (Day 1 and 22 of treatment) following daily oral administration of ANS 9801 to pregnant female rabbits

Day 6

Dose level (mg/kg/day)	C_{max} (ng/ml)	T_{max} (hours)	C_{24} (ng/ml)	AUC_{24} (ng.h/ml)
500	10513	0.5	3447	151896
1000	13694	1	4134	117112
2000	18923	1	6693	274417

Day 27

Dose level (mg/kg/day)	C_{max} (ng/ml)	T_{max} (hours)	C_{24} (ng/ml)	AUC_{24} (ng.h/ml)
500	14338	1	2581	147414
1000	21382	1	8356	268999
2000	30400	1	20563	486206

FIGURE 1

Mean plasma concentrations of ANS 9801 on Day 6 after mating (Day 1 of treatment) following daily oral administration of ANS 9801 to pregnant female rabbits

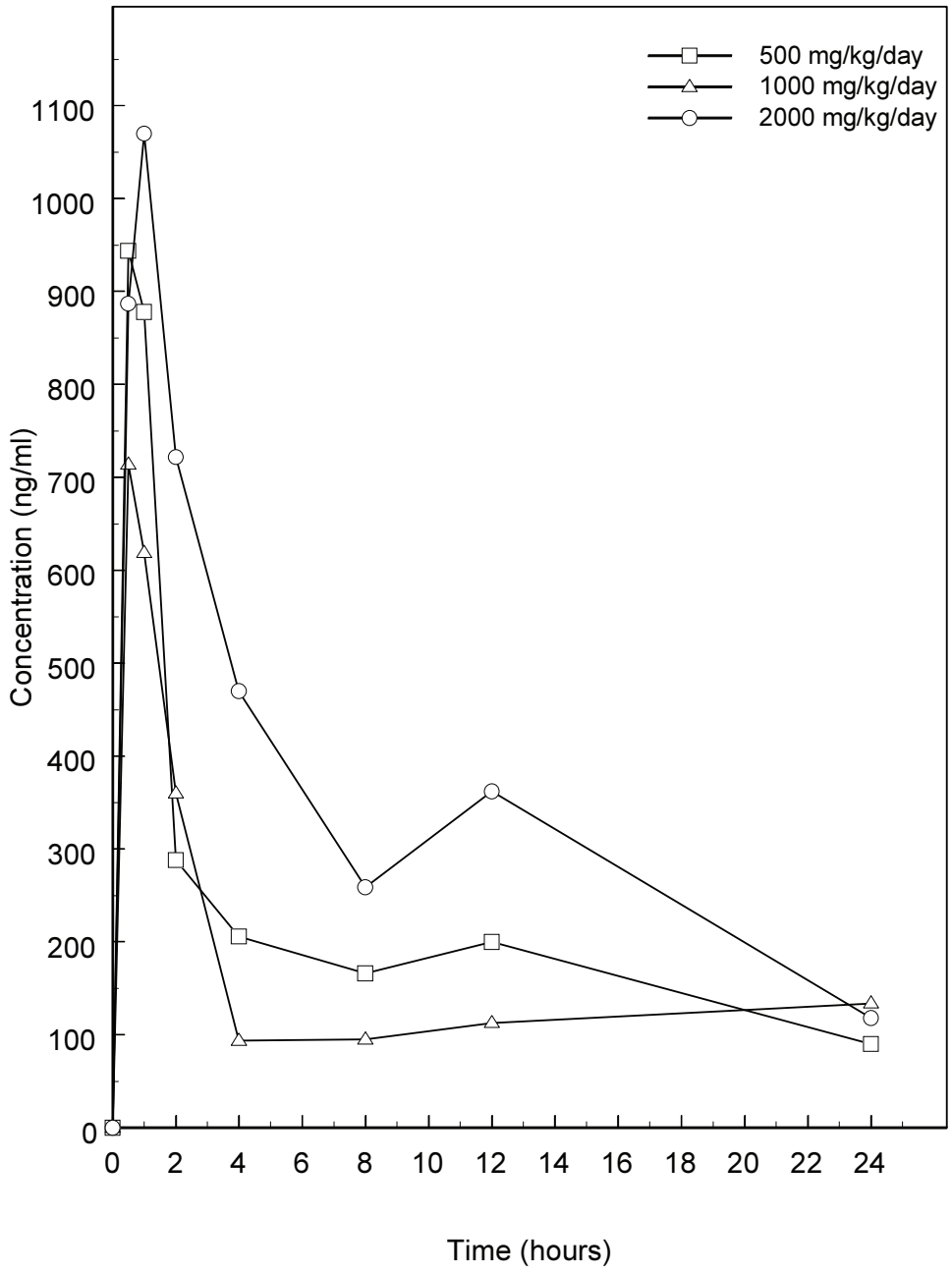


FIGURE 2

Mean plasma concentrations of ANS 9801 on Day 27 after mating (Day 22 of treatment) following daily oral administration of ANS 9801 to pregnant female rabbits

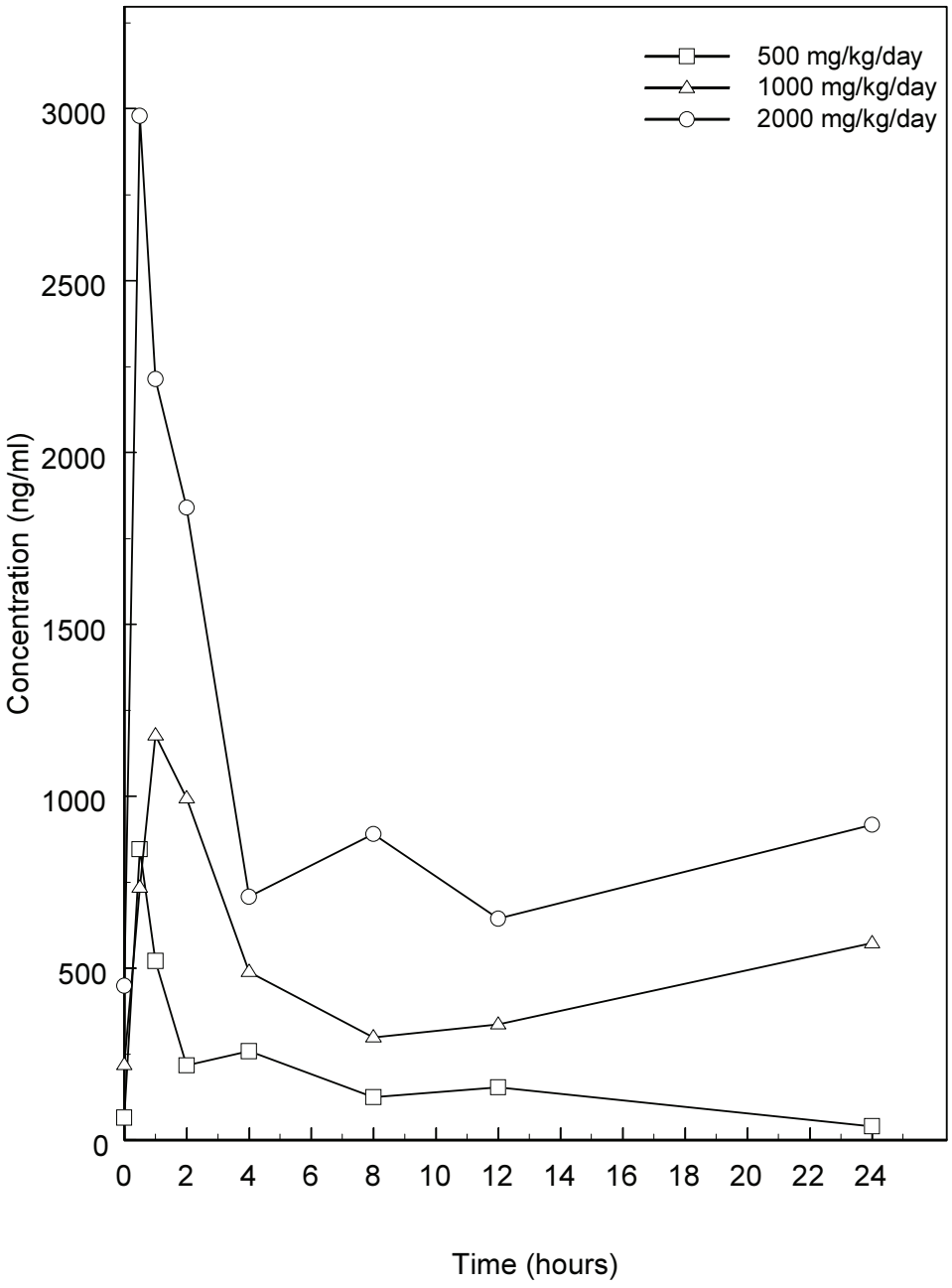


FIGURE 3

Mean plasma concentrations of ANS 9801-acid on Day 6 after mating (Day 1 of treatment) following daily oral administration of ANS 9801 to pregnant female rabbits

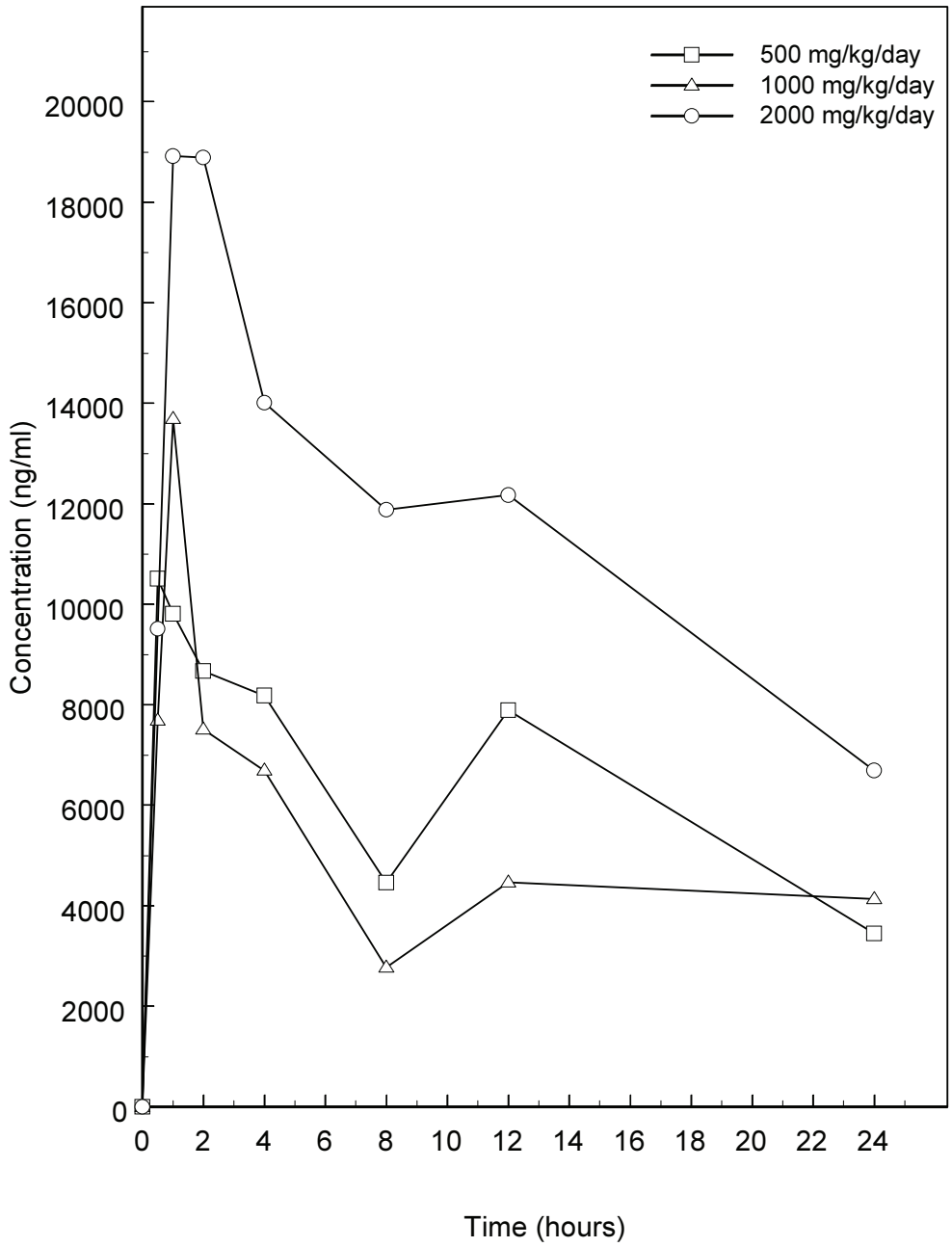


FIGURE 4

Mean plasma concentrations of ANS 9801-acid on Day 27 after mating (Day 22 of treatment) following daily oral administration of ANS 9801 to pregnant female rabbits

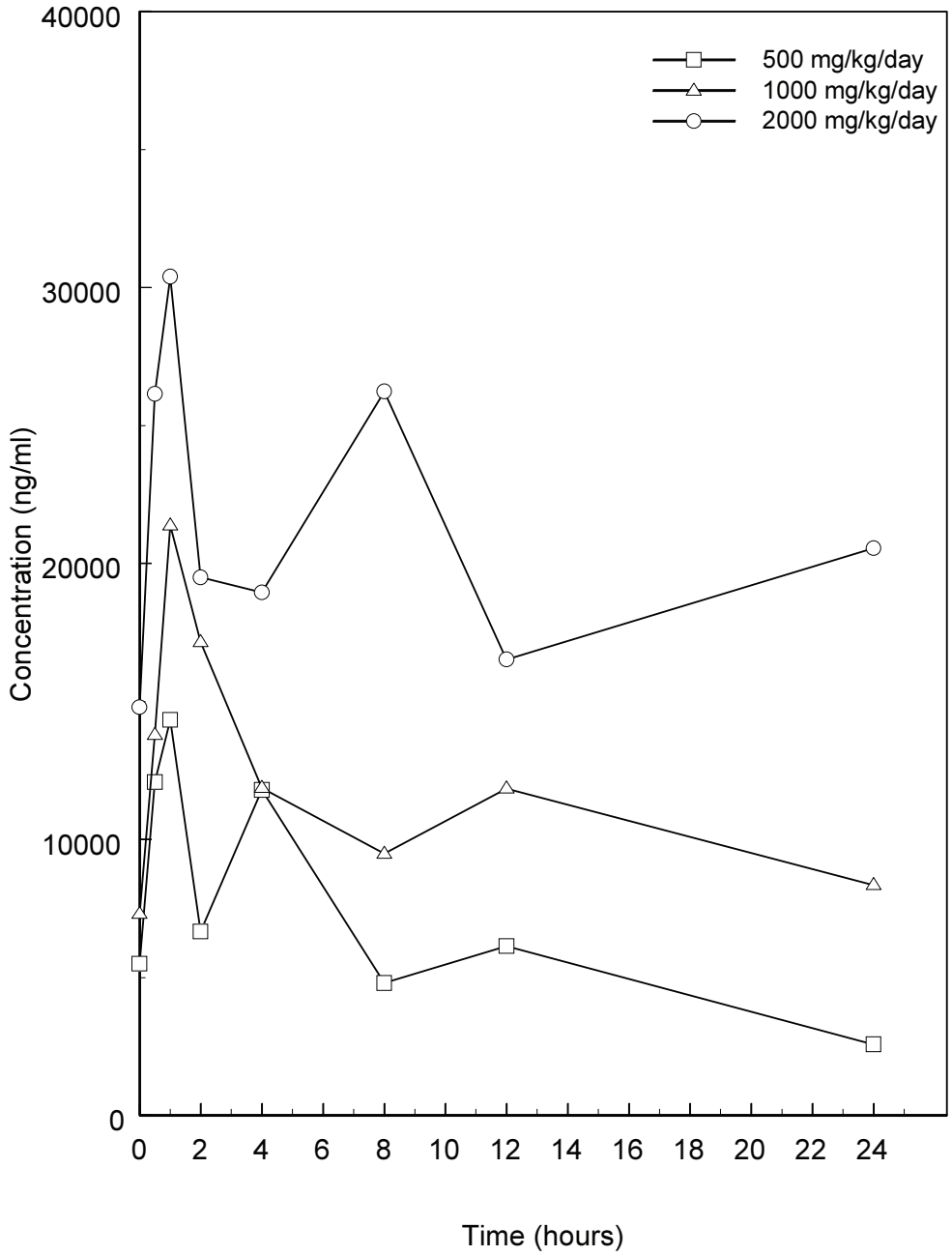


FIGURE 5

Relationship between maximum mean plasma concentrations (C_{max}) of ANS 9801 and dose level on Day 6 and Day 27 after mating (Days 1 and 22 of treatment) following daily oral administration of ANS 9801 to pregnant female rabbits

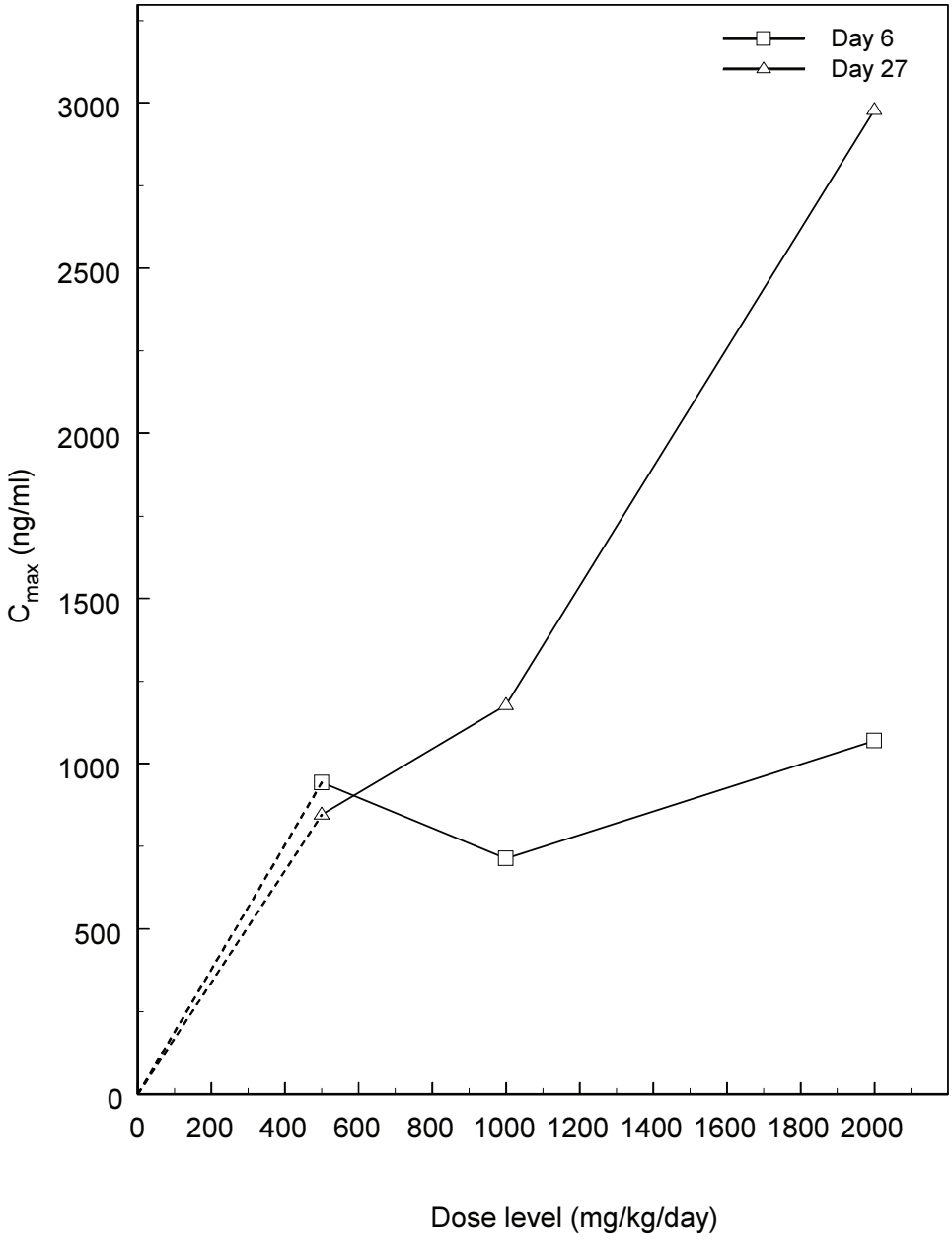


FIGURE 6

Relationship between areas under the mean plasma ANS 9801 concentration-time curves (AUC_{24}) and dose level on Day 6 and Day 27 after mating (Days 1 and 22 of treatment) following daily oral administration of ANS 9801 to pregnant female rabbits

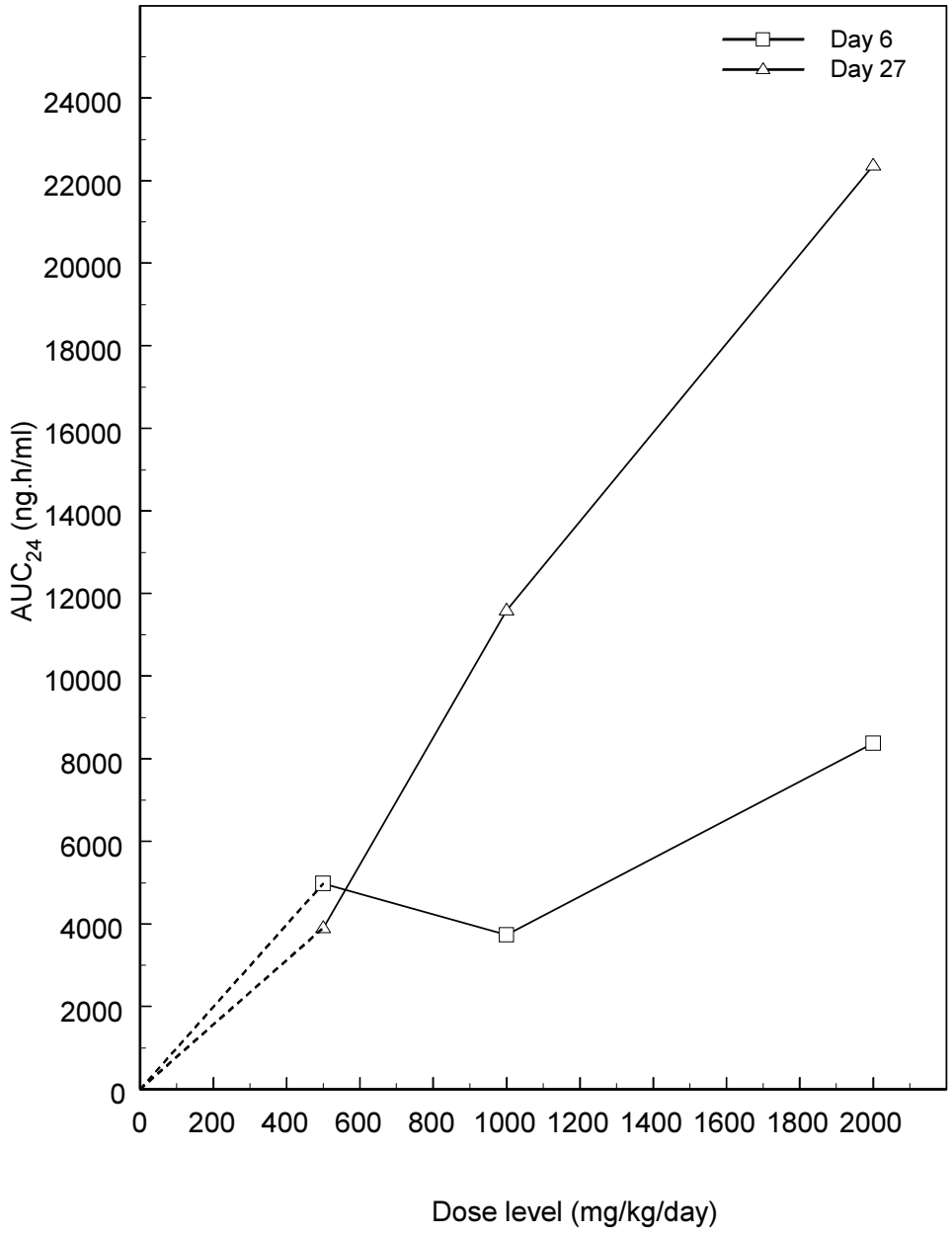


FIGURE 7

Maximum mean plasma concentrations (C_{max}) of ANS 9801 and areas under the mean plasma ANS 9801 concentration-time curves (AUC_{24}), each adjusted to a dose level of 1 mg/kg/day, on Days 6 and 27 after mating (Days 1 and 22 of treatment) following daily oral administration of ANS 9801 to pregnant female rabbits

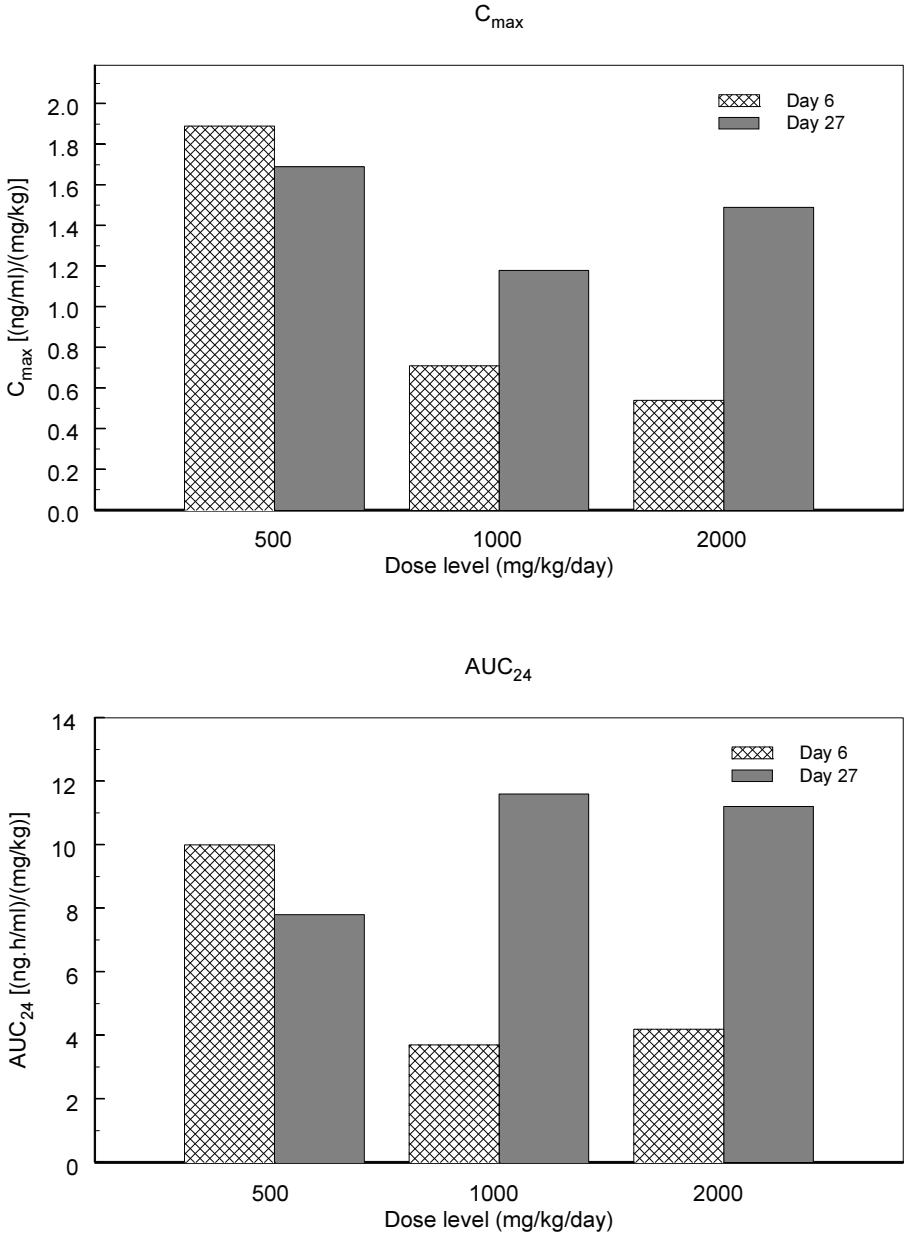


FIGURE 8

Relationship between maximum mean plasma concentrations (C_{max}) of ANS 9801-acid and dose level on Day 6 and Day 27 after mating (Days 1 and 22 of treatment) following daily oral administration of ANS 9801 to pregnant female rabbits

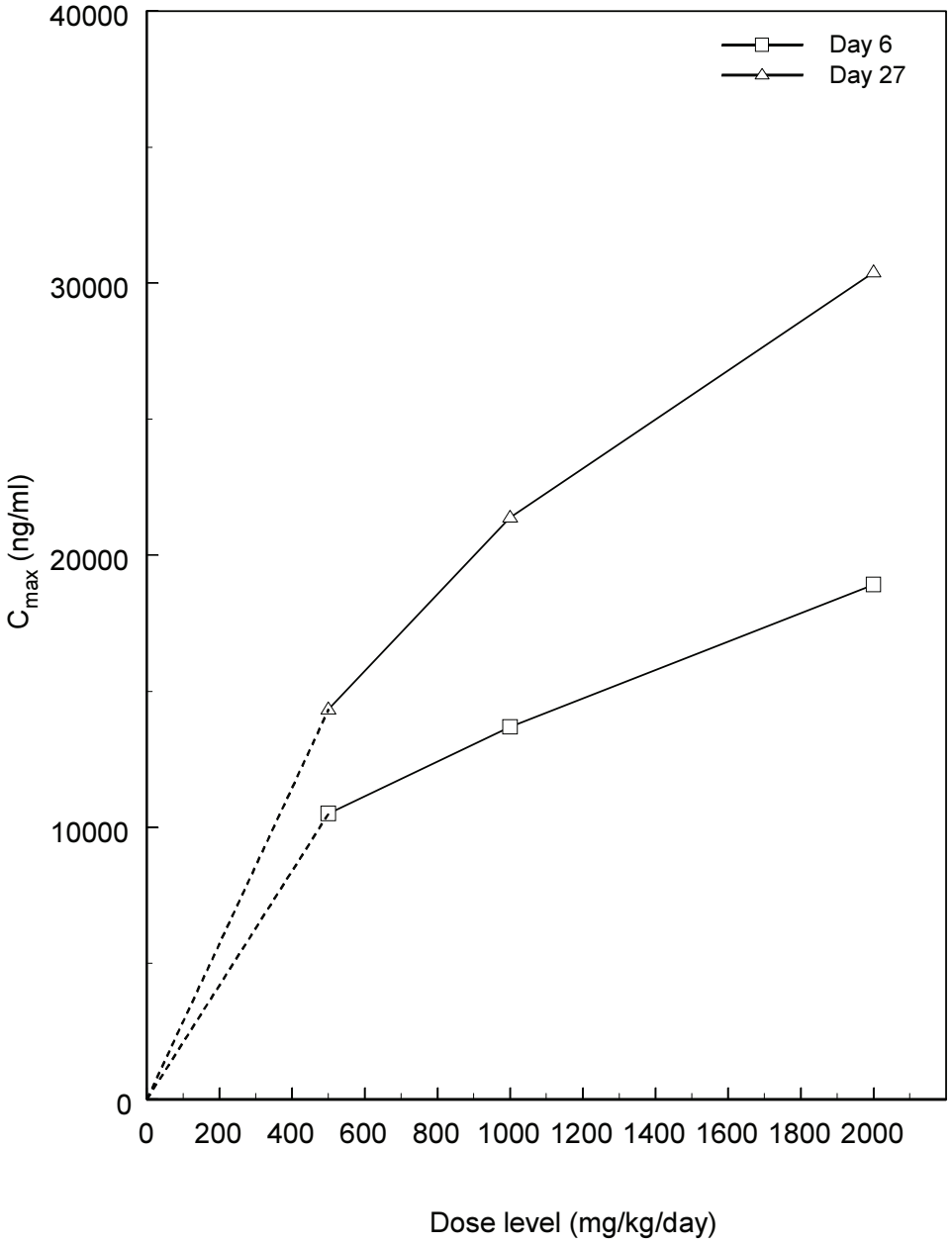


FIGURE 9

Relationship between areas under the mean plasma ANS 9801-acid concentration-time curves (AUC_{24}) and dose level on Day 6 and Day 27 after mating (Days 1 and 22 of treatment) following daily oral administration of ANS 9801 to pregnant female rabbits

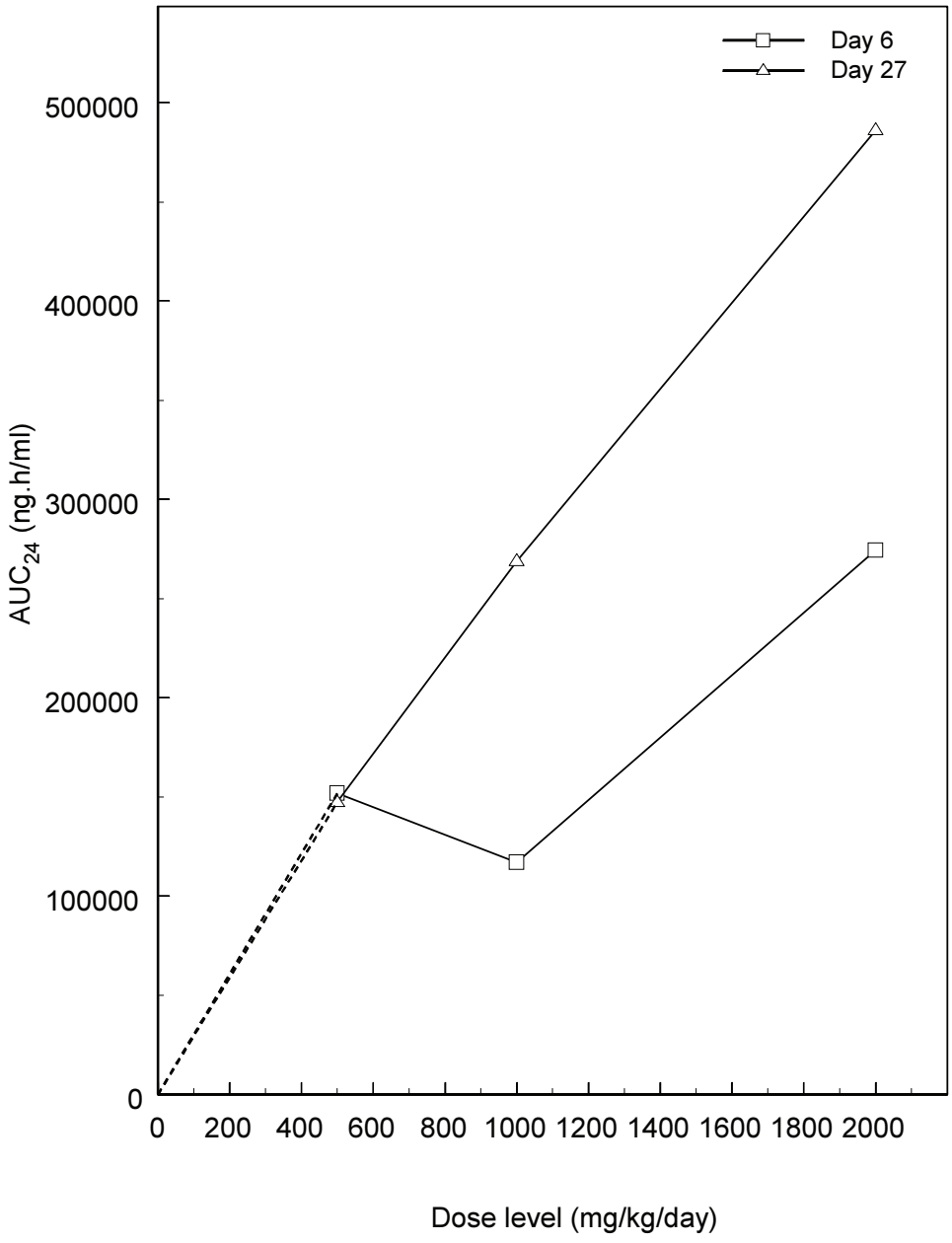
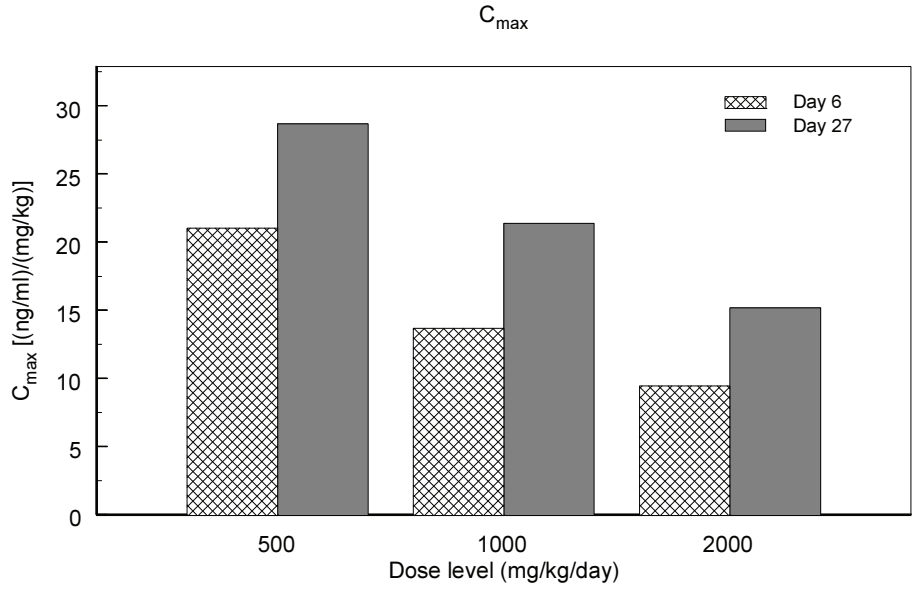
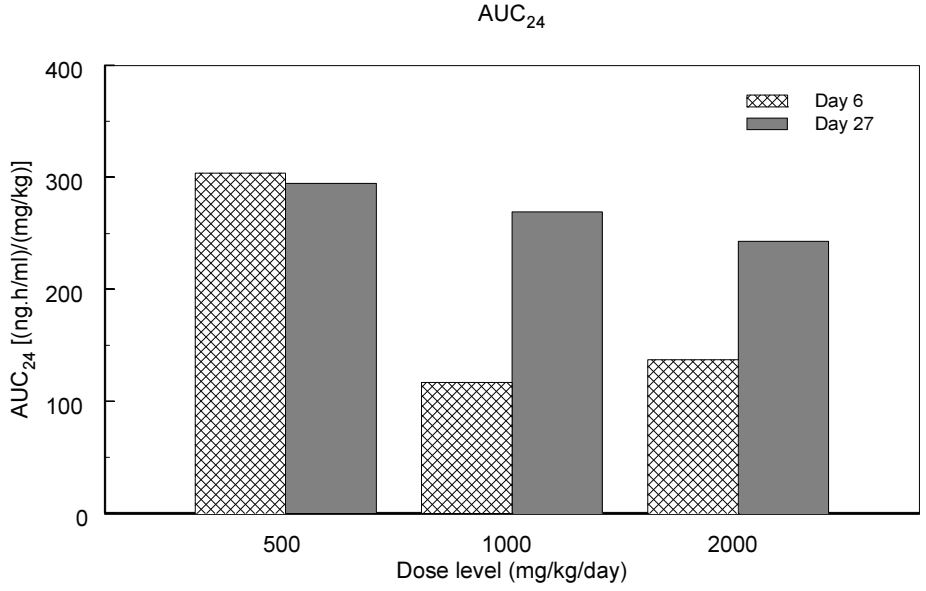


FIGURE 10

Maximum mean plasma concentrations (C_{max}) of ANS 9801-acid and areas under the mean plasma ANS 9801-acid concentration-time curves (AUC_{24}), each adjusted to a dose level of 1 mg/kg/day, on Days 6 and 27 after mating (Days 1 and 22 of treatment) following daily oral administration of ANS 9801 to pregnant female rabbits





APPENDIX 1

Statistical Analysis of AUC_{24} and C_{max}

INTRODUCTION

Plasma concentrations of ANS 9801 and the metabolite, ANS 9801-acid, were available over 24 hours for female rabbits on Days 6 and 27 of pregnancy. There were three groups receiving ANS 9801 by oral gavage at dose levels of 500, 1000 and 2000 mg/kg/day.

METHODS

For each group, in order to obtain estimates of areas under the plasma concentration-time curves to 24 hours (AUC_{24}), given that different animals were used to obtain blood samples at each time point, the trapezoidal approach was used on the mean values at each time point. Variances were also obtained using standard statistical techniques (Bailer 1988) allowing comparisons between groups to be carried out. It should be noted that the animals were in fact bled four times on each day of sampling. However, the results are considered to be reliable as the correlations between observations on the same animal are believed to be low.

The means and their variances were dose adjusted to 1 mg/kg, using mean achieved intakes, and chi-square tests for heterogeneity (Miller *et al* 1939) were carried out between the dose-adjusted estimates for the three dose groups, a measure of dose proportionality on each day. Comparisons were also made between the days for each dose group separately using chi-square tests.

The plasma concentrations which gave the maximum mean concentration of ANS 9801 (maxC) were used as estimates of C_{max}. Analysis of variance was carried out on the dose-adjusted values with dose group, time and their interaction as factors. The significance level of the dose effect, for the dose-adjusted variable, reflects the degree of dose proportionality. A logarithmic transformation was performed on the maxC data prior to analysis in order to stabilise the variances. Missing values were estimated within the analyses by the method of least squares. A similar analysis was performed on the maximum mean concentrations of the metabolite.

DATA HANDLING

The data were received electronically and analysed using SAS 8.2 (SAS Institute Inc., 1999) for AUC_{24} and Genstat version 5.3.2 (Payne *et al* 1993) for maxC.

APPENDIX 1

(Statistical analysis of AUC_{24} and Cmax – continued)

RESULTS

The mean AUC_{24} for ANS 9801 and the significance levels for comparisons between them have been presented in Table 1. The unadjusted mean AUC_{24}s and their standard errors have also been presented for completeness in Table 2. The results for the metabolite have been presented in Tables 3 and 4.

The analyses of variance and tables of means for maxC are given in Tables 5 and 6 for ANS 9801 and the metabolite respectively.

For AUC_{24} of ANS 9801, there was evidence of significant non-proportionality on both Day 6 and Day 27 ($p \leq 0.002$). The Day 27 mean values were found to be significantly higher than the Day 6 values at both the 1000 and 2000 mg/kg/day dose levels ($p < 0.001$). However, there was some evidence of a decrease in mean values from Day 6 to Day 27 at the 500 mg/kg dose levels ($p = 0.034$).

For AUC_{24} of the metabolite there was evidence of significant non-proportionality on Day 6 only ($p < 0.001$). The Day 27 mean values were found to be significantly higher than the Day 6 values at both the 1000 and 2000 mg/kg/day dose levels ($p \leq 0.003$).

For maxC of both ANS 9801 and the metabolite, ANS 9801-acid, there was evidence of significant non-proportionality ($p \leq 0.030$). The mean values on Day 27 were found to be significantly higher than on Day 6 ($p \leq 0.038$).

REFERENCES

BAILER, A.J. (1988) Testing for the equality of area under the curves when using destructive measurement techniques *J. Pharmacokin. Biopharm.,* 16, 303-309.

MILLER, L.C., BLISS, C.I. and BRAUN, H.A. (1939) The assay of digitalis *J. Amer. Pharm. Ass.* 28, 644-657.

SAS Institute (1999) SAS OnlineDoc® *Version Eight*. SAS Institute Inc., Cary, NC, USA

PAYNE, R.W. *et al* (1993) *Genstat 5 Release 3 Reference Manual.* Clarendon Press, Oxford.

KEY TO TABLES

df	Degrees of freedom
mv	Missing values
ss	Sums of squares
ms	Mean square
vr	Variance ratio
F pr	F ratio probability
Dose	ANS 9801 (mg/kg/day)

APPENDIX 1

(Statistical analysis of AUC_{24} and Cmax – continued)

TABLE 1

Areas under the ANS 9801 plasma concentration-time curves to 24 hours (ng.h/ml) - scaled to 1 mg/kg

Dose (mg/kg/day)	Day 6		Day 27		Time Difference	
	Mean	Variance	Mean	Variance	χ^2	*p*-value
500	9.96	0.554	7.81	0.477	4.49	0.034
1000	3.74	0.161	11.59	5.060	11.83	<0.001
2000	4.19	0.768	11.18	0.451	40.09	<0.001
Non-proportionality	χ^2=55.27	*p*<0.001	χ^2=13.04	*p*=0.002		

TABLE 2

Areas under the ANS 9801 plasma concentration-time curves to 24 hours (ng.h/ml)

Dose (mg/kg/day)	Day 6		Day 27	
	Mean	se	Mean	se
500	4982	372	3906	345
1000	3737	401	11594	2249
2000	8382	1753	22367	1343

APPENDIX 1

(Statistical analysis of AUC_{24} and Cmax – continued)

TABLE 3

Areas under the metabolite[#] plasma concentration-time curves to 24 hours (ng.h/ml) - scaled to 1 mg/kg

Dose (mg/kg/day)	Day 6		Day 27		Time Difference	
	Mean	Variance	Mean	Variance	χ^2	*p*-value
500	304	239	295	801	0.08	0.78
1000	117	109	269	2442	9.04	0.003
2000	137	197	243	372	19.71	<0.001
Non-proportionality	χ^2=105.42	*p*<0.001	χ^2=2.31	*p*=0.31		

TABLE 4

Areas under the metabolite[#] plasma concentration-time curves to 24 hours (ng.h/ml)

Dose (mg/kg/day)	Day 6		Day 27	
	Mean	se	Mean	se
500	151896	7733	147414	14152
1000	117112	10460	268999	49419
2000	274417	28082	486206	38571

[#] ANS 9801-acid

APPENDIX 1

(Statistical analysis of AUC_{24} and Cmax – continued)

TABLE 5

Maximum value of the mean plasma concentration of ANS 9801 – scaled to 1 mg/kg

Analysis of variance (logarithmically transformed data)

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr.
Dose	2	1.8252	0.9126	6.10	0.025
Time	1	1.0296	1.0296	6.88	0.031
Dose x Time	2	0.8749	0.4374	2.92	0.112
Residual	8(4)	1.1976	0.1497		
Total	13(4)	4.1669			

Table of means (back-transformed - ng/ml)

Dose	500 mg	1000 mg	2000 mg
	1.714	0.863	0.883

Time	Day 6	Day 27
	0.860	1.388

Time	Day 6	Day 27
Dose		
500 mg	1.740	1.688
1000 mg	0.699	1.064
2000 mg	0.523	1.488

APPENDIX 1

(Statistical analysis of AUC_{24} and Cmax – continued)

TABLE 6

Maximum value of the mean plasma concentration of the metabolite[#] – scaled to 1 mg/kg

Analysis of variance (logarithmically transformed data)

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr.
Dose	2	1.4851	0.7425	4.90	0.030
Time	1	0.8445	0.8445	5.58	0.038
Dose x Time	2	0.0125	0.0063	0.04	0.960
Residual	11(1)	1.6660	0.1515		
Total	16(1)	3.9718			

Table of means (back-transformed - ng/ml)

Dose	500 mg	1000 mg	2000 mg
	23.68	15.66	11.76

Time	Day 6	Day 27
	13.16	20.29

Time	Day 6	Day 27
Dose		
500 mg	19.66	28.53
1000 mg	12.66	19.38
2000 mg	9.16	15.11

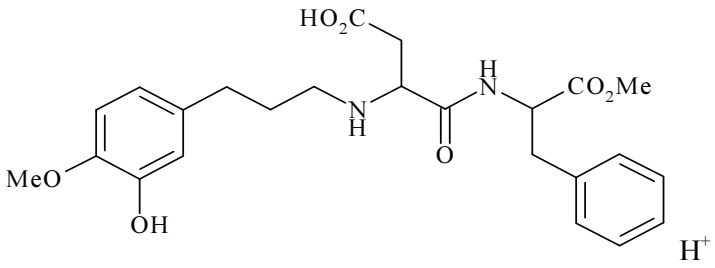
[#] ANS 9801-acid

APPENDIX 2

Mass Spectrometry of ANS 9801 and ANS 9801-acid

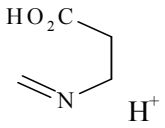
FIGURE 1

Proposed fragmentation of ANS 9801



m/z = 459

Collision activated dissociation
using nitrogen.
Collision energy = *ca.* 30 eV



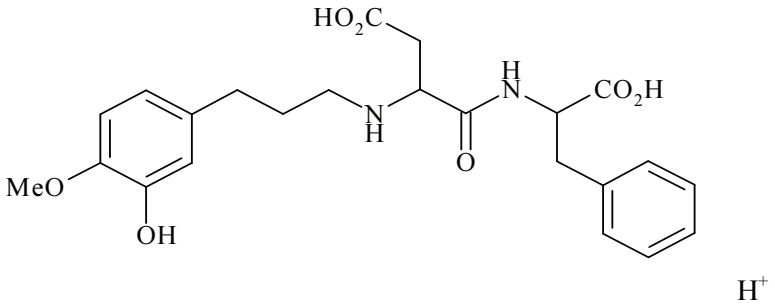
m / z = 102

APPENDIX 2

(Mass spectrometry of ANS 9801 and ANS 9801-acid - continued)

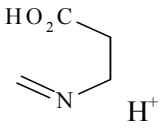
FIGURE 2

Proposed fragmentation of ANS 9801-acid



m/z = 445

Collision activated dissociation
using nitrogen.
Collision energy = *ca.* 30 eV



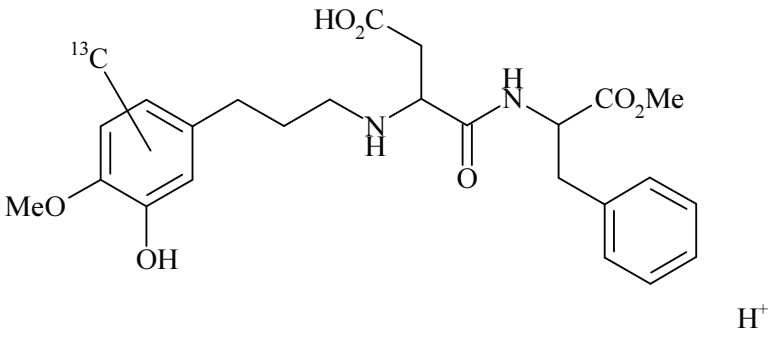
m / z = 102

APPENDIX 2

(Mass spectrometry of ANS 9801 and ANS 9801-acid - continued)

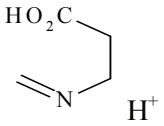
FIGURE 3

Proposed fragmentation of ^{13}C-ANS 9801



m/z = 465

Collision activated dissociation
using nitrogen.
Collision energy = *ca.* 30 eV



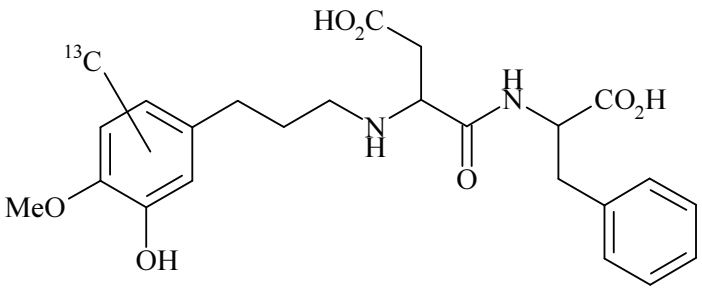
m / z = 102

APPENDIX 2

(Mass spectrometry of ANS 9801 and ANS 9801-acid - continued)

FIGURE 4

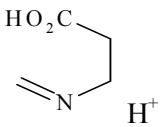
Proposed fragmentation of ^{13}C-ANS 9801-acid



H^+

m/z = 451

Collision activated dissociation
using nitrogen.
Collision energy = *ca.* 30 eV



H^+

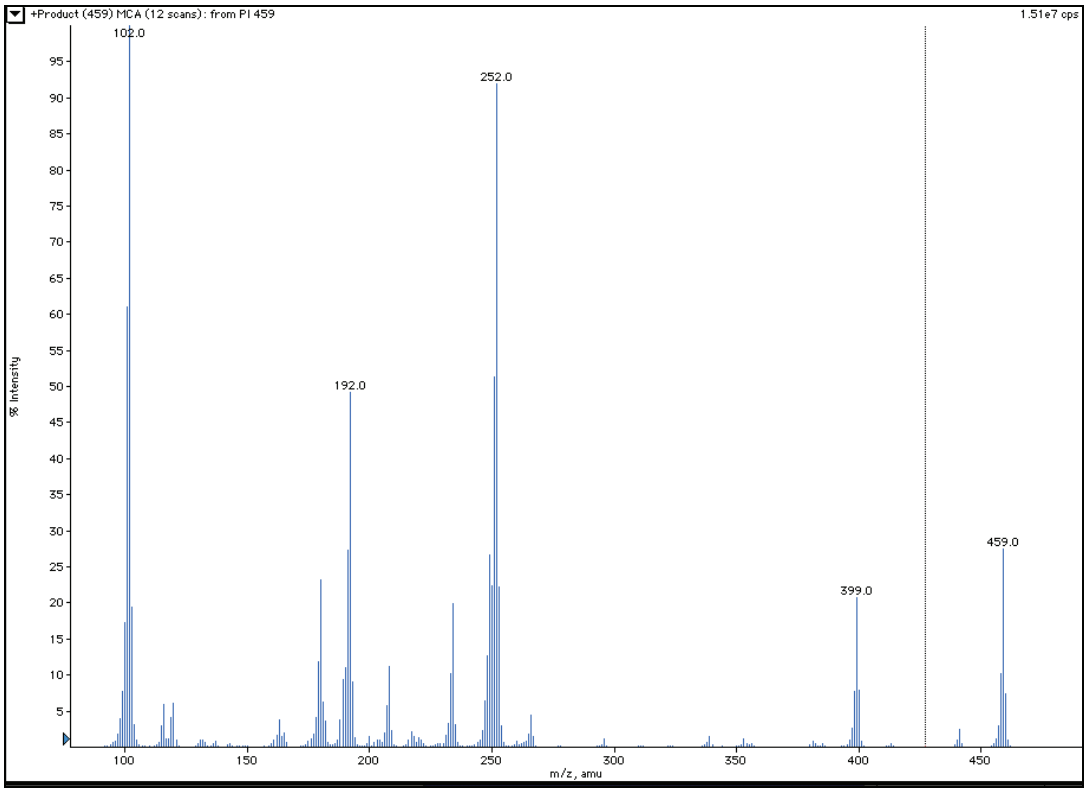
m / z = 102

APPENDIX 2

(Mass spectrometry of ANS 9801 and ANS 9801-acid - continued)

FIGURE 5

Product ion mass spectrum of ANS 9801
(from quasi-molecular ion at m/z 459)

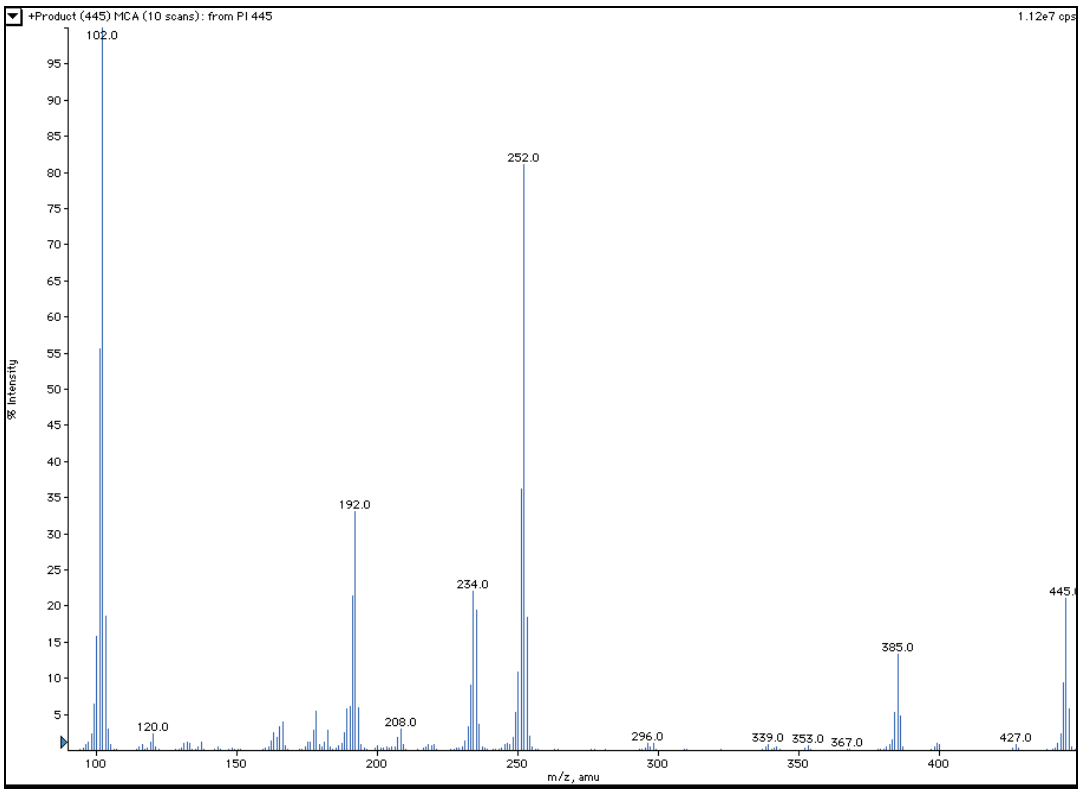


APPENDIX 2

(Mass spectrometry of ANS 9801 and ANS 9801-acid - continued)

FIGURE 6

Product ion mass spectrum of ANS 9801-acid
(from quasi-molecular ion at m/z 445)

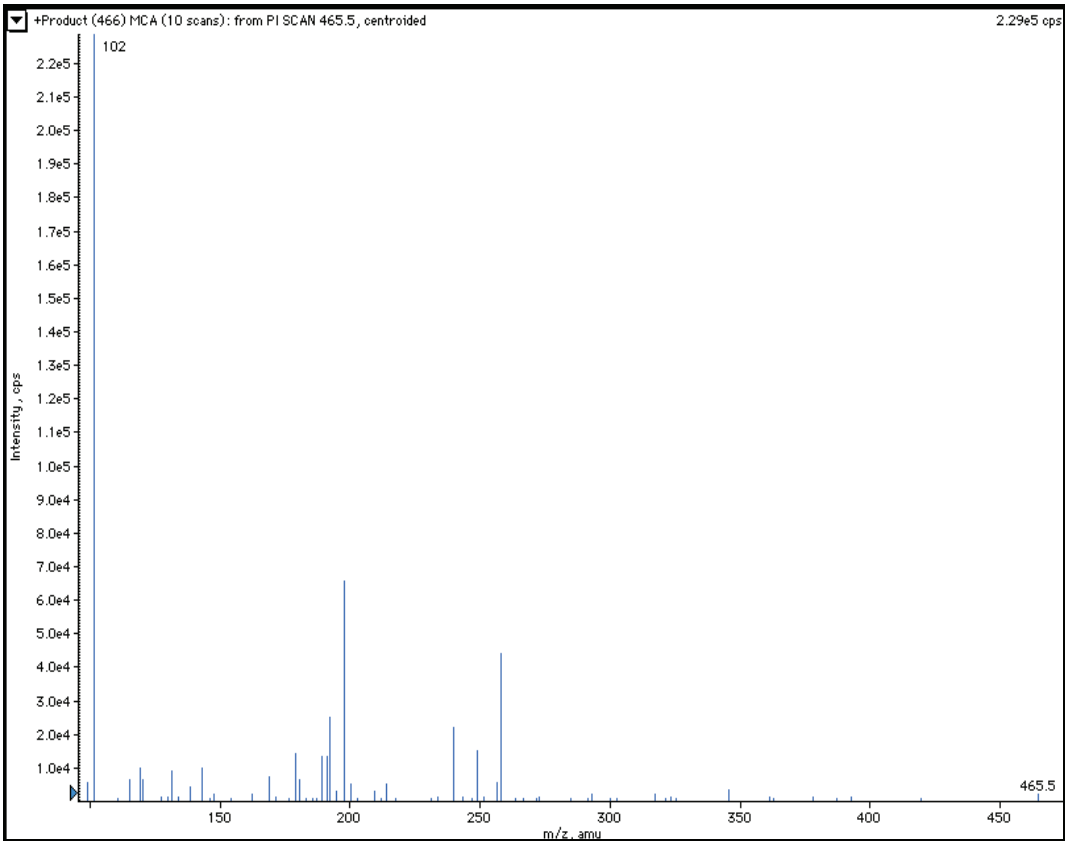


APPENDIX 2

(Mass spectrometry of ANS 9801 and ANS 9801-acid - continued)

FIGURE 7

Product ion mass spectrum of ^{13}C-ANS 9801
(from quasi-molecular ion at m/z 465)

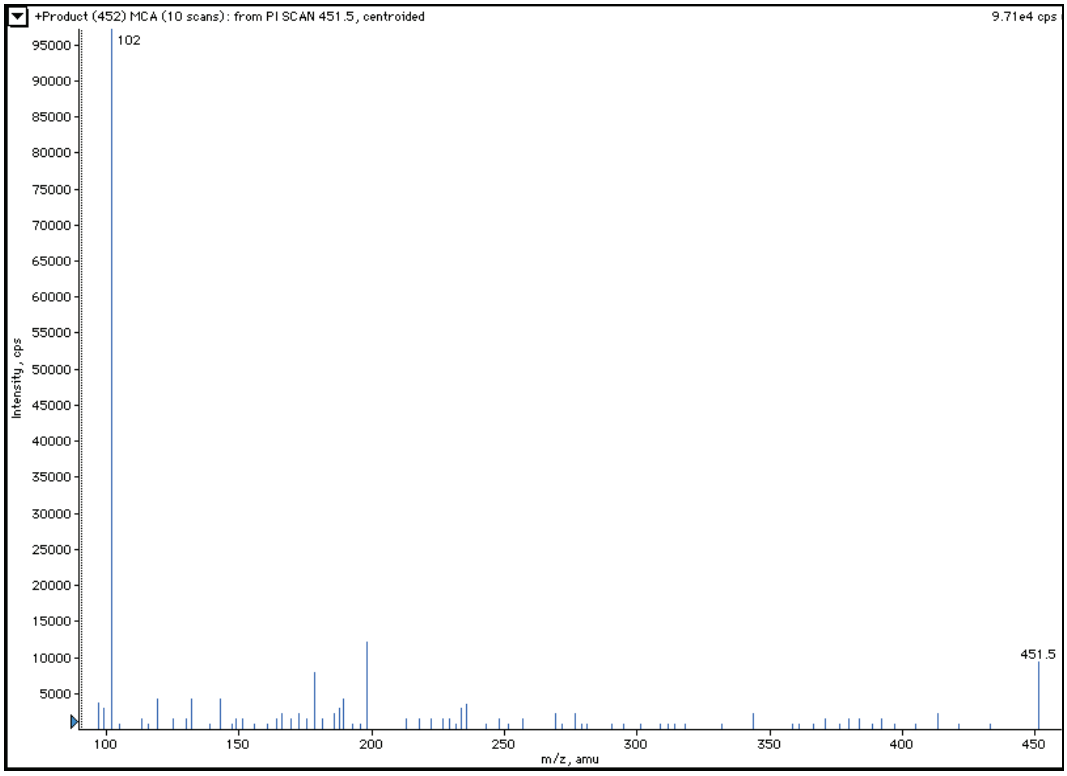


APPENDIX 2

(Mass spectrometry of ANS 9801 and ANS 9801-acid - continued)

FIGURE 8

Product ion mass spectrum of ^{13}C-ANS 9801-acid (from quasi-molecular ion at m/z 451)



APPENDIX 3

Bioanalytical Parameters

TABLE 1

Summary of calibration line data for assay batches 1-9 (ANS 9801)

Batch number	Slope	Intercept	Correlation coefficient	Instrument number
1	0.0226	-0.00145	0.9989	4
2	0.0359	-0.000391	0.9973	6
3	0.0388	0.00339	0.9992	6
4	0.0403	-0.000181	0.9998	6
5	0.0380	-0.000306	0.9998	6
6	0.0356	-0.00138	0.9995	6
7	0.0374	-0.000653	0.9996	6
8	0.0323	-0.00159	0.9996	6
9	0.0396	0.00164	0.9998	6
Mean	0.0356	-	-	-
sd	0.00544	-	-	-
CV(%)	15.3	-	-	-

sd Standard deviation.
CV Coefficient of variation.
Mean, sd and CV were calculated using unrounded data.

APPENDIX 3

(Bioanalytical parameters - continued)

TABLE 2

Summary of calibration line data for assay batches 1-7 (ANS 9801-acid)

Batch number	Slope	Intercept	Correlation coefficient	Instrument number
1	0.0233	-0.000748	0.9983	4
2	0.0250	0.00302	0.9972	6
3	0.0312	-0.00683	0.9973	6
4	0.0275	0.000067	0.9997	6
5	0.0251	-0.00114	0.9998	6
7	0.0232	-0.00464	0.9989	6
Mean	0.0259	-	-	-
sd	0.00303	-	-	-
CV(%)	11.7	-	-	-

sd Standard deviation.
CV Coefficient of variation.
Mean, sd and CV were calculated using unrounded data.

APPENDIX 3

(Bioanalytical parameters - continued)

TABLE 3

Inter-batch accuracy of calibration standards for ANS 9801

Batch number	Calibration standard concentration (ng/ml)							
	0.500	**1.00**	**2.00**	**5.00**	**10.0**	**20.0**	**50.0**	**100**
1	0.491	1.08	2.40*	5.18	9.07	20.7	47.3	103
2	0.528	1.10	2.01	4.99	8.73	20.7	45.7	106
3	0.453	1.02	2.05	5.24	9.37	22.0	48.6	100
4	0.476	1.12	1.97	4.86	10.2	19.3	50.0	101
5	0.481	1.09	1.82	5.35	9.70	20.2	50.6	99.3
6	0.556	0.987	2.09	4.85	9.53	18.6	49.9	102
7	0.536	1.04	1.95	4.86	9.89	19.1	48.9	102
8	0.542	0.988	2.00	4.96	9.88	18.9	49.0	102
9	0.582	0.953	1.91	4.90	10.0	19.3	51.0	100
Mean	0.516	1.04	1.98	5.02	9.60	19.9	49.0	102
sd	0.0427	0.0597	0.0827	0.187	0.474	1.11	1.68	1.99
CV	8.27	5.71	4.18	3.73	4.94	5.60	3.43	1.95
RE	3.22	4.42	-1.20	0.414	-4.02	-0.678	-1.99	1.72
n	9	9	8	9	9	9	9	9

sd Standard deviation.
CV Coefficient of variation.
RE Relative error.
n Number of replicates
* Standard outside acceptance criteria, not included in the statistical calculations.
Mean, sd, CV and RE were calculated using unrounded data.

APPENDIX 3

(Bioanalytical parameters - continued)

TABLE 4

Inter-batch accuracy of calibration standards for ANS 9801-acid

Batch number	Calibration standard concentration (ng/ml)							
	0.500	**1.00**	**2.00**	**5.00**	**10.0**	**20.0**	**50.0**	**100**
1	0.465	1.11	1.98	5.51	8.68	21.8	47.6	102
2	0.536	0.972	2.56*	4.97	10.1	20.7	44.6	106
3	0.800*	1.14	2.62*	5.30	8.63	19.5	46.5	106
4	0.569	1.03	1.94	4.58	10.1	19.3	49.7	101
5	0.592	0.971	1.86	4.88	9.96	19.3	49.4	102
7	0.725*	1.14	2.15	4.44	9.79	18.4	49.3	103
Mean	0.540	1.06	1.98	4.95	9.54	19.8	47.8	103
sd	0.0553	0.0787	0.1247	0.410	0.697	1.21	2.03	1.99
CV	10.2	7.43	6.29	8.28	7.31	6.12	4.25	1.93
RE	8.07	5.93	-0.917	-1.08	-4.60	-0.796	-4.30	3.24
n	4	6	4	6	6	6	6	6

sd Standard deviation.
CV Coefficient of variation.
RE Relative error.
n Number of replicates
* Standard outside acceptance criteria, not included in the statistical calculations.
Mean, sd, CV and RE were calculated using unrounded data.

APPENDIX 3

(Bioanalytical parameters - continued)

TABLE 5

Summary of quality control data for ANS 9801

Batch number	QCLOW (1.5 ng/ml)		QCMED (7 ng/ml)		QCHIGH (70 ng/ml)	
1	1.49	1.59	7.20	6.94	75.9	70.6
2	1.58	1.48	7.08	6.82	72.7	71.1
3	1.52	1.35	6.96	7.02	74.9	74.7
4	1.60	1.63	7.16	7.27	79.2	74.7
5	1.59	1.42	7.04	6.46	68.9	68.4
6	1.70	1.64	7.00	7.14	75.0	73.5
7	1.77	1.40	6.43	6.67	70.3	67.8
8	1.68	1.54	7.17	7.01	74.7	75.3
9	1.56	1.49	6.80	7.15	75.0	74.8
Mean	1.56		6.96		73.2	
sd	0.108		0.243		3.04	
CV	6.92		3.48		4.16	
RE	3.82		-0.553		4.56	

sd Standard deviation.
CV Coefficient of variation.
RE Relative error.
Mean, sd, CV and RE were calculated using unrounded data.

APPENDIX 3

(Bioanalytical parameters - continued)

TABLE 6

Summary of quality control data for ANS 9801-acid

Batch number	QCLOW (1.5 ng/ml)		QCMED (7 ng/ml)		QCHIGH (70 ng/ml)	
1	**1.13**	1.51	7.36	7.18	79.7	73.6
2	1.25	1.46	6.76	6.69	68.9	68.3
3	1.57	1.50	7.14	7.08	71.2	65.1
4	1.55	1.53	**8.62**	7.67	75.1	74.1
5	1.46	1.39	7.00	6.53	69.6	69.5
7	1.46	1.40	6.96	7.41	70.5	68.2
Mean	1.43		7.20		71.1	
sd	0.128		0.551		3.91	
CV	8.94		7.66		5.49	
RE	-4.38		2.86		1.63	

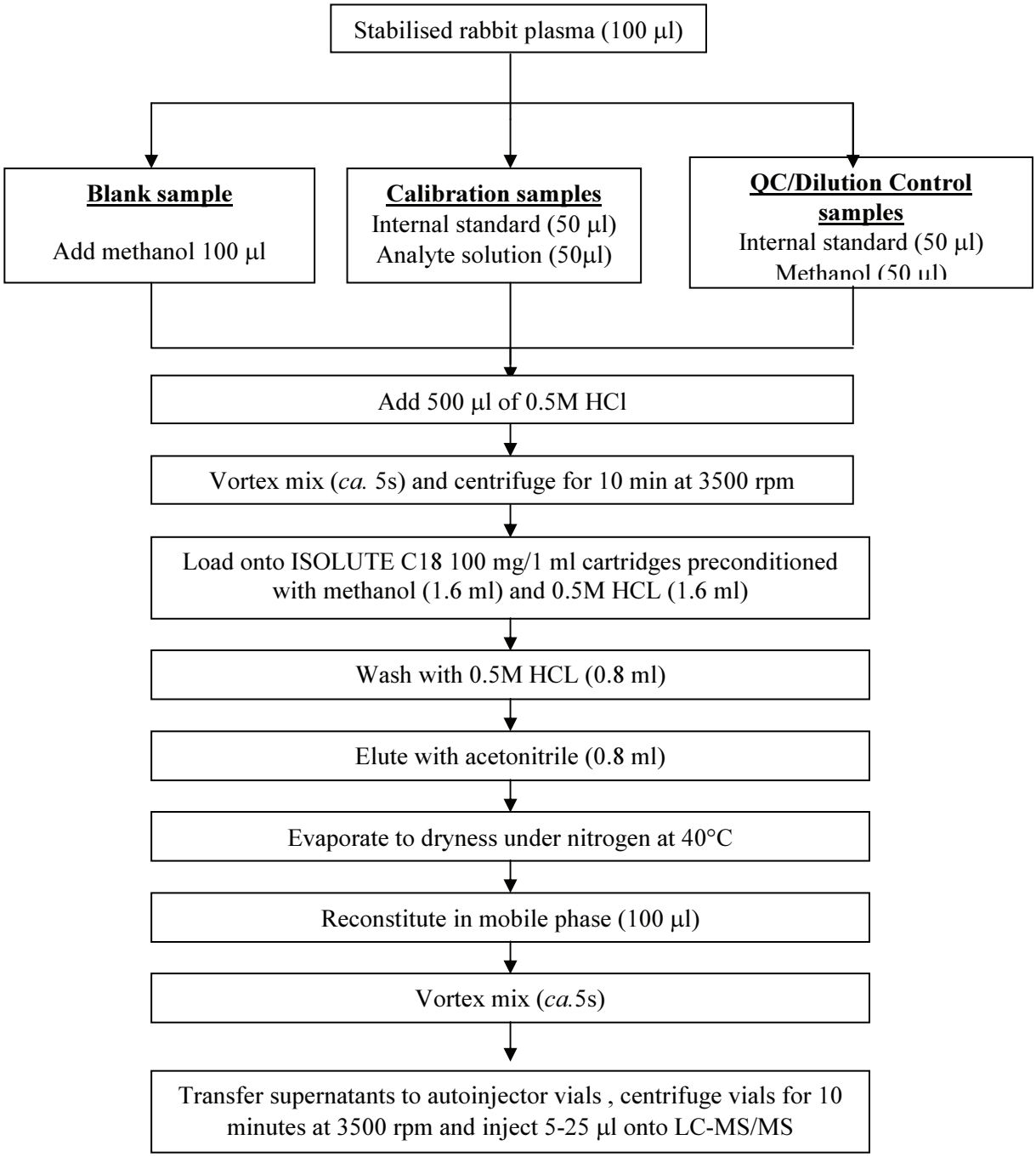
sd Standard deviation.
CV Coefficient of variation.
RE Relative error.
Mean, sd, CV and RE were calculated using unrounded data.
Values in bold were outside the criteria of acceptance of ± 15% (± 20% at QC LOW) but were included in the statistical analysis.

APPENDIX 3

(Bioanalytical parameters - continued)

FIGURE 1

Schematic representation of the sample extraction procedure



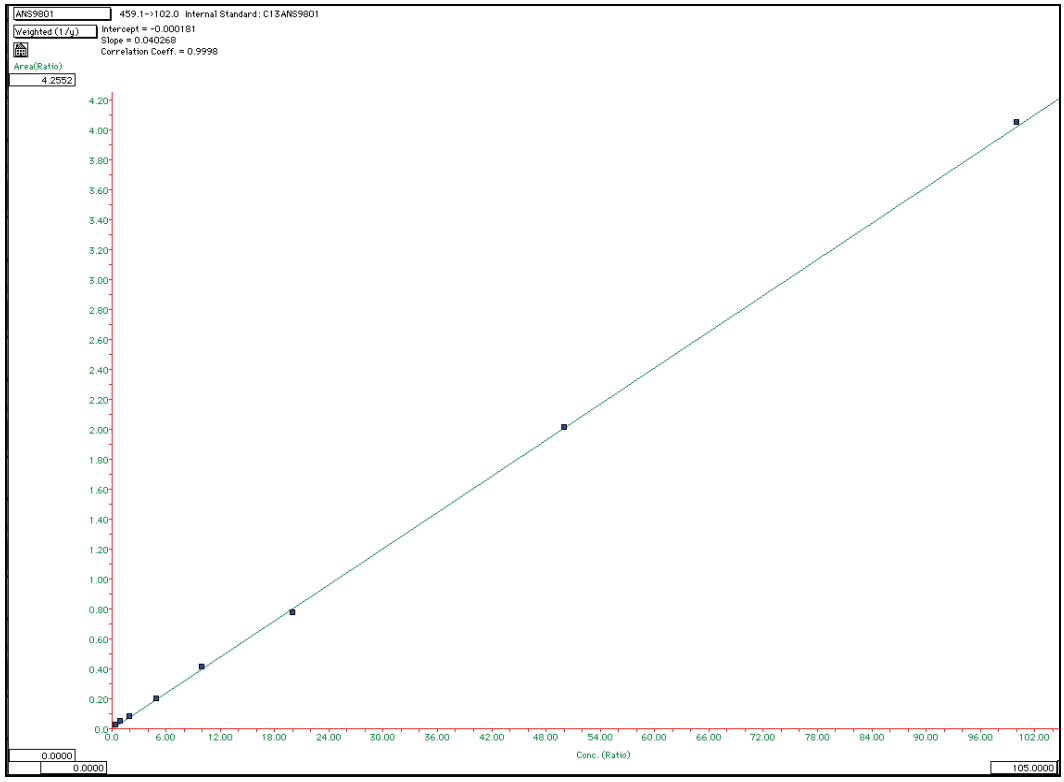
APPENDIX 3

(Bioanalytical parameters - continued)

FIGURE 2

Representative Calibration Line for ANS 9801

(Batch 4)



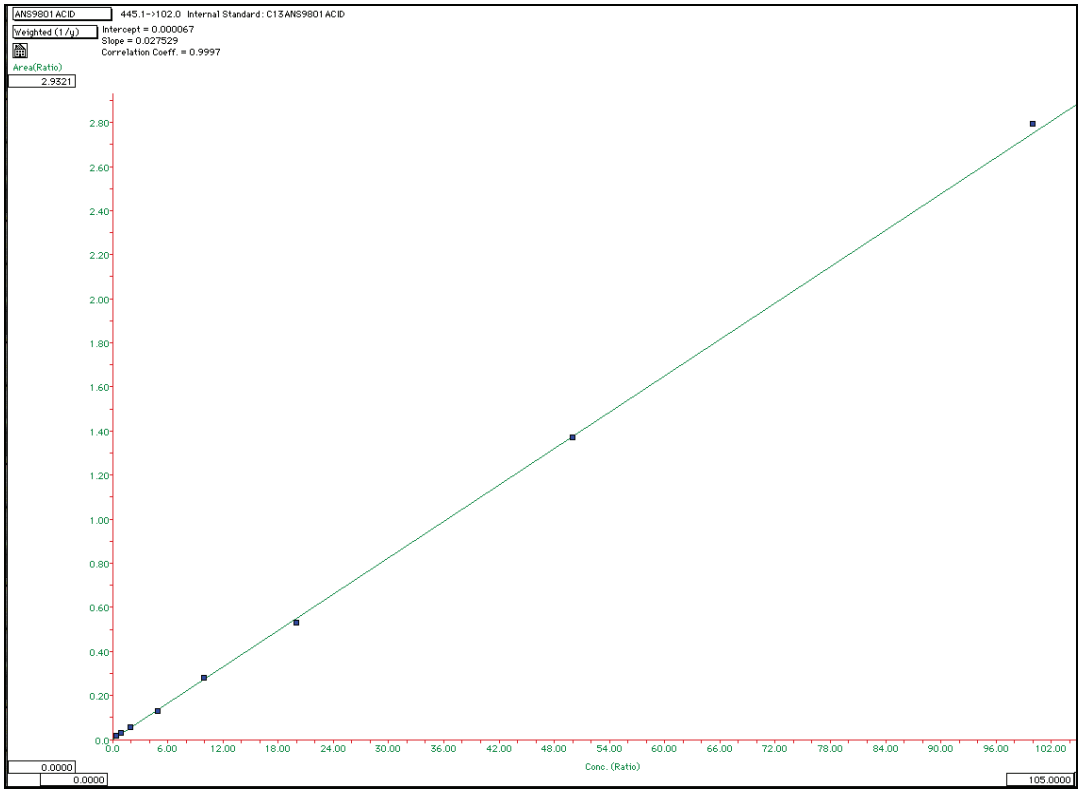
APPENDIX 3

(Bioanalytical parameters - continued)

FIGURE 3

Representative Calibration Line for ANS 9801-acid

(Batch 4)



APPENDIX 3

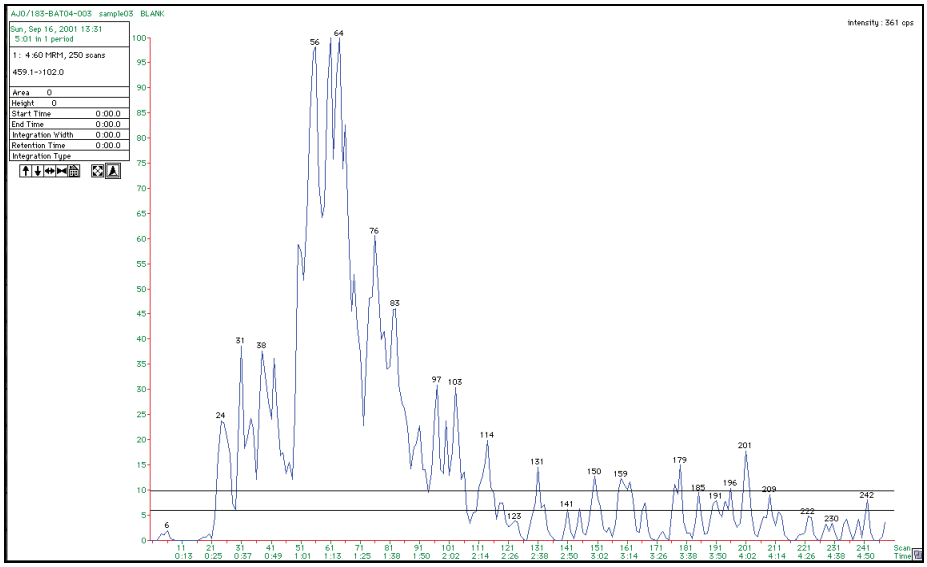
(Bioanalytical parameters - continued)

FIGURE 4

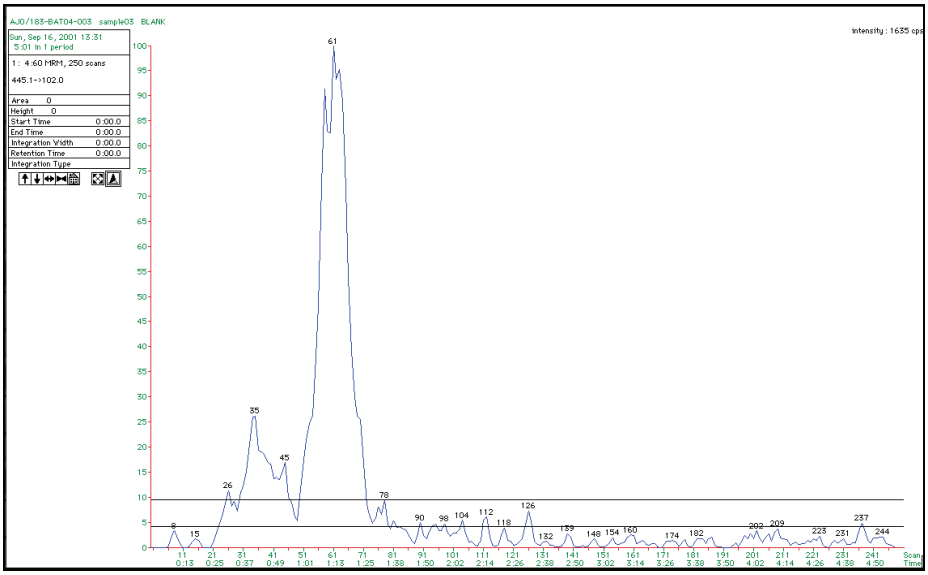
Representative chromatograms

Extracted Blank sample

ANS 9801



ANS 9801-acid



APPENDIX 3

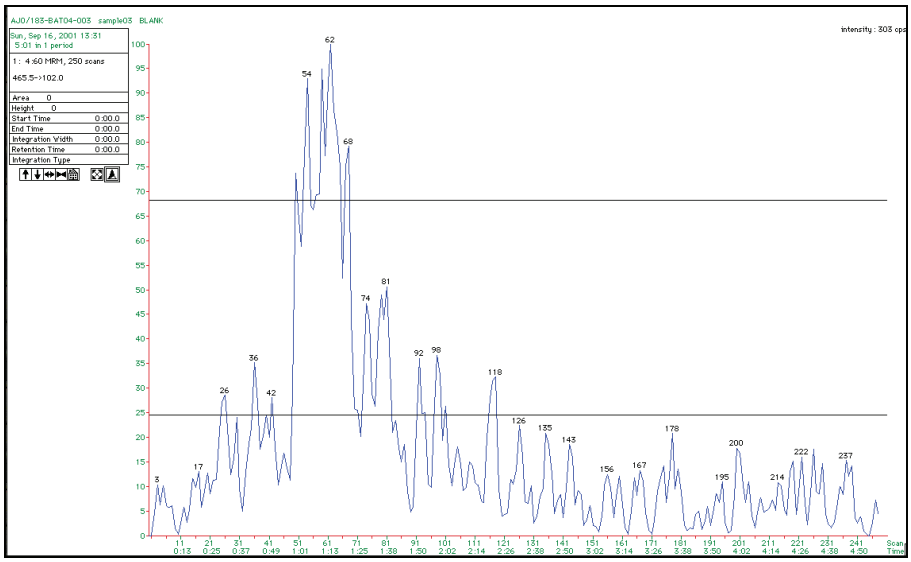
(Bioanalytical parameters - continued)

FIGURE 4

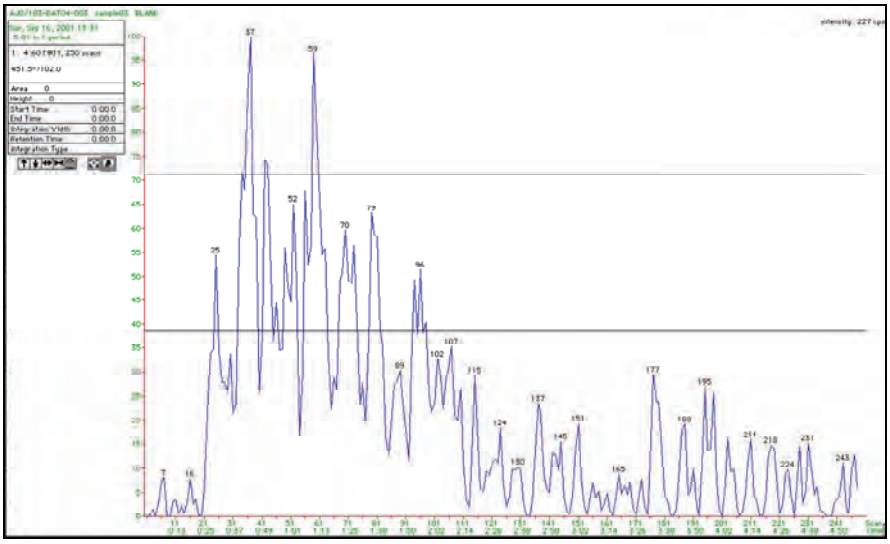
Representative chromatograms

Extracted Blank sample- continued

^{13}C-ANS 9801



^{13}C-ANS 9801-acid



APPENDIX 3

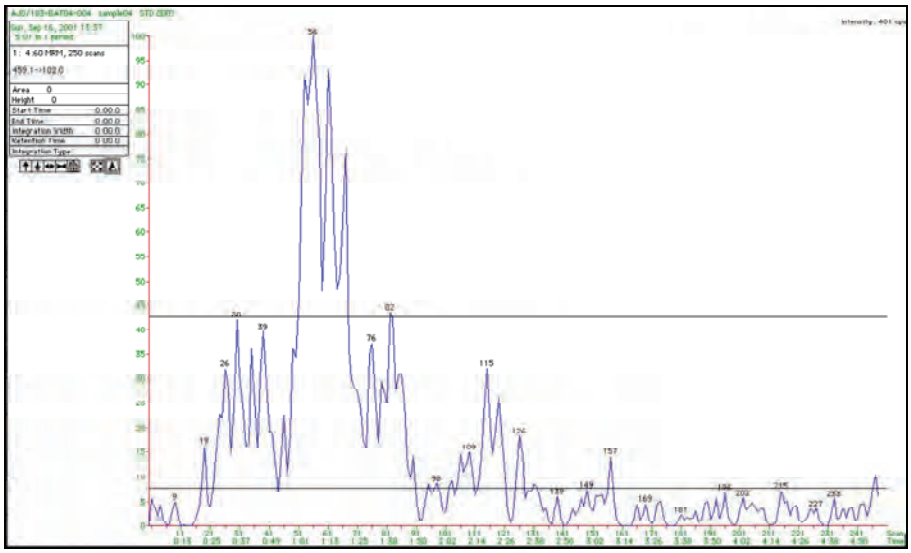
(Bioanalytical parameters - continued)

FIGURE 5

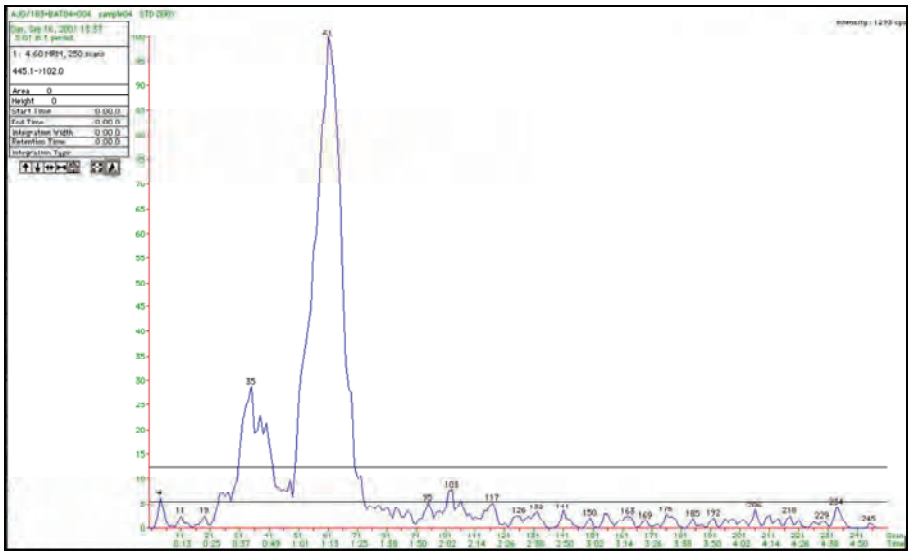
Representative chromatograms

Calibration Standard 0 ng/ml

ANS 9801



ANS 9801-acid



APPENDIX 3

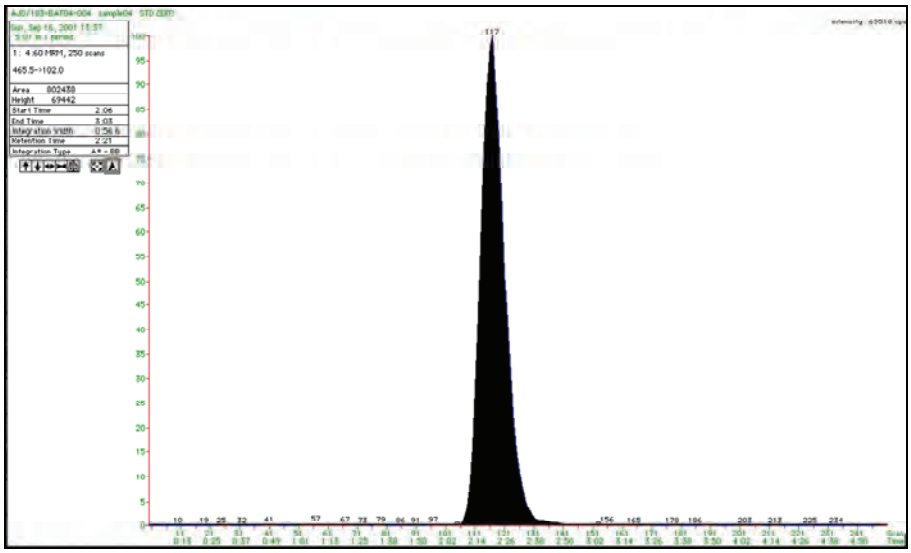
(Bioanalytical parameters - continued)

FIGURE 5

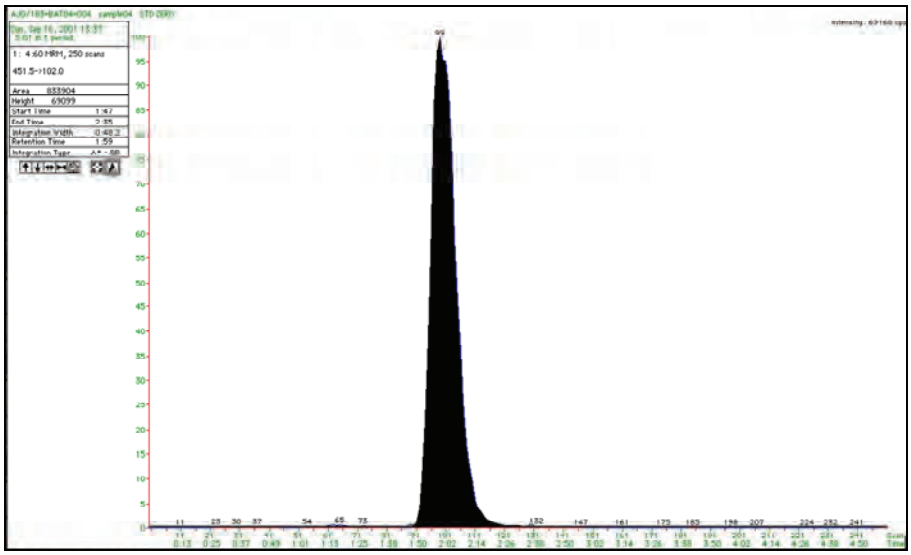
Representative chromatograms

Calibration Standard 0 ng/ml- continued

^{13}C-ANS 9801



^{13}C-ANS 9801-acid



APPENDIX 3

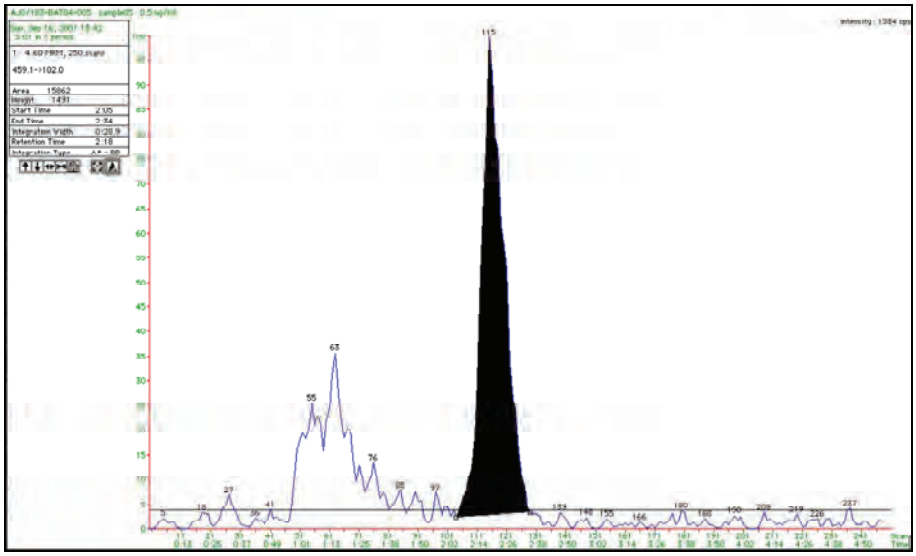
(Bioanalytical parameters - continued)

FIGURE 6

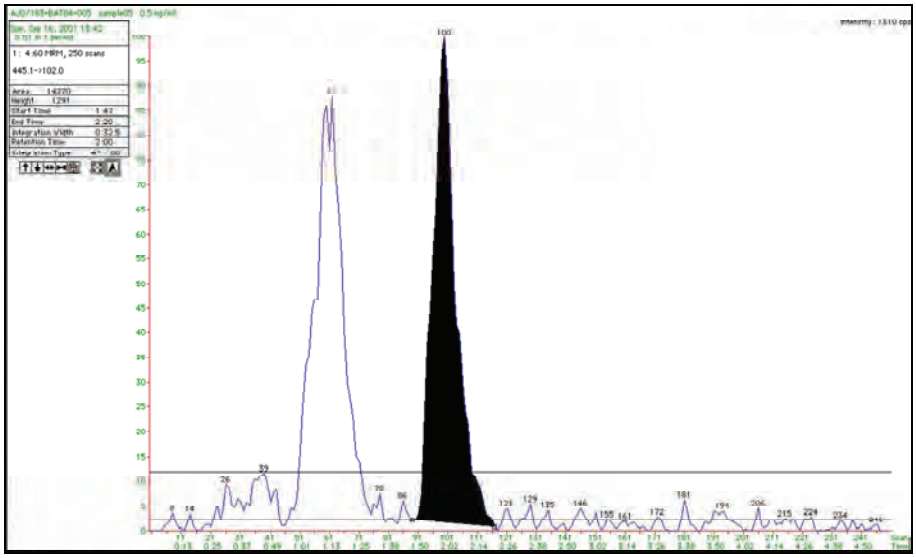
Representative chromatograms

Calibration Standard 0.5 ng/ml (LLOQ)

ANS 9801



ANS 9801-acid



APPENDIX 3

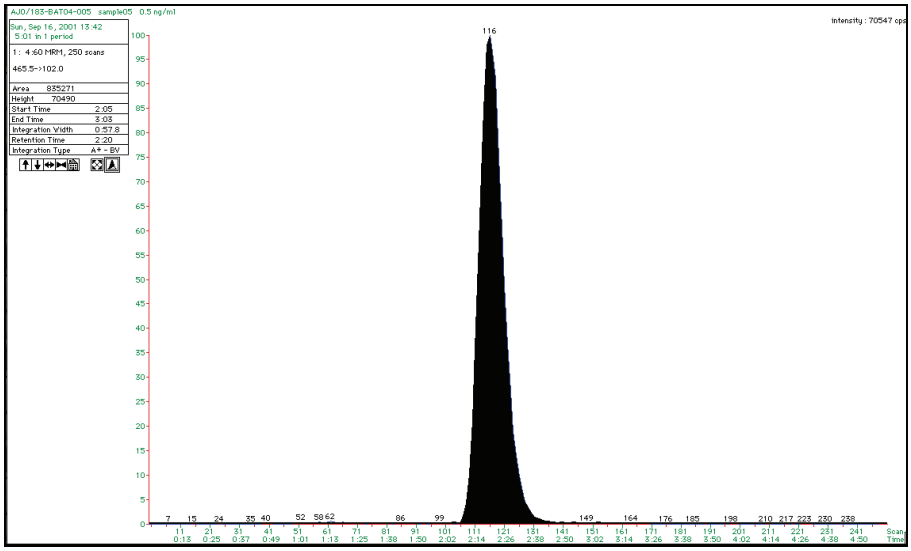
(Bioanalytical parameters - continued)

FIGURE 6

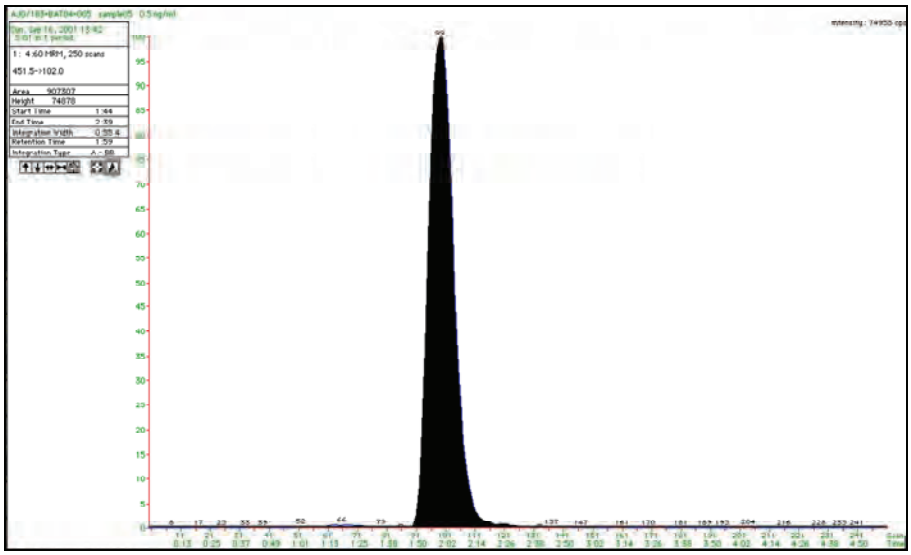
Representative chromatograms

Calibration Standard 0.5 ng/ml (LLOQ)- continued

^{13}C-ANS 9801



^{13}C-ANS 9801-acid



APPENDIX 3

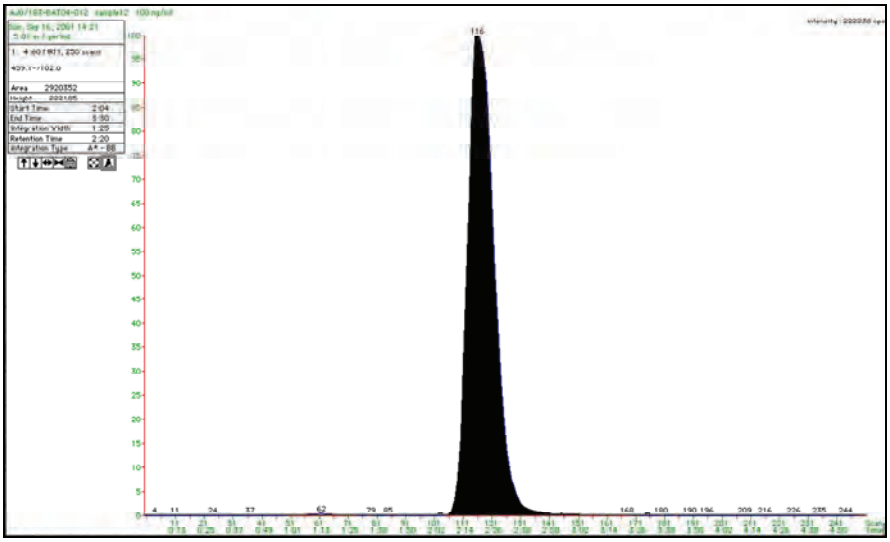
(Bioanalytical parameters – continued)

FIGURE 7

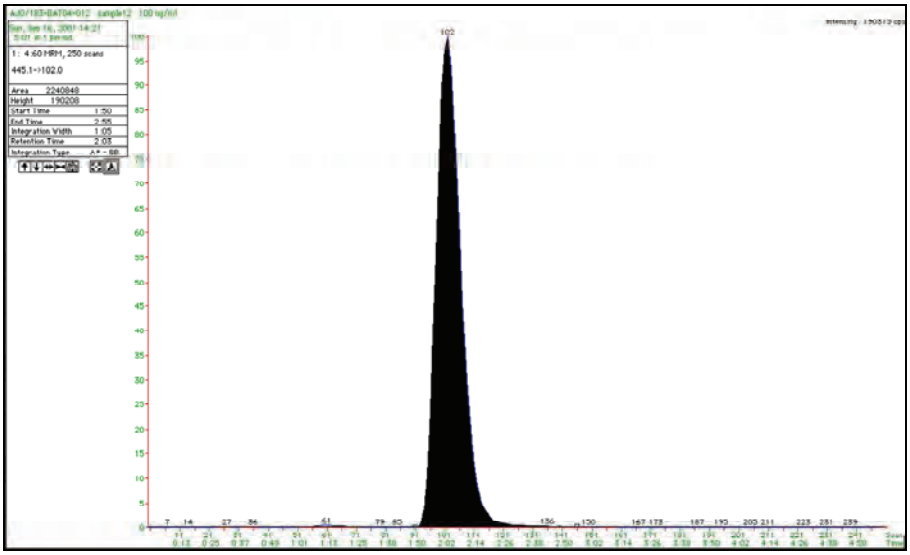
Representative chromatograms

Calibration Standard 100 ng/ml

ANS 9801



ANS 9801-acid



APPENDIX 3

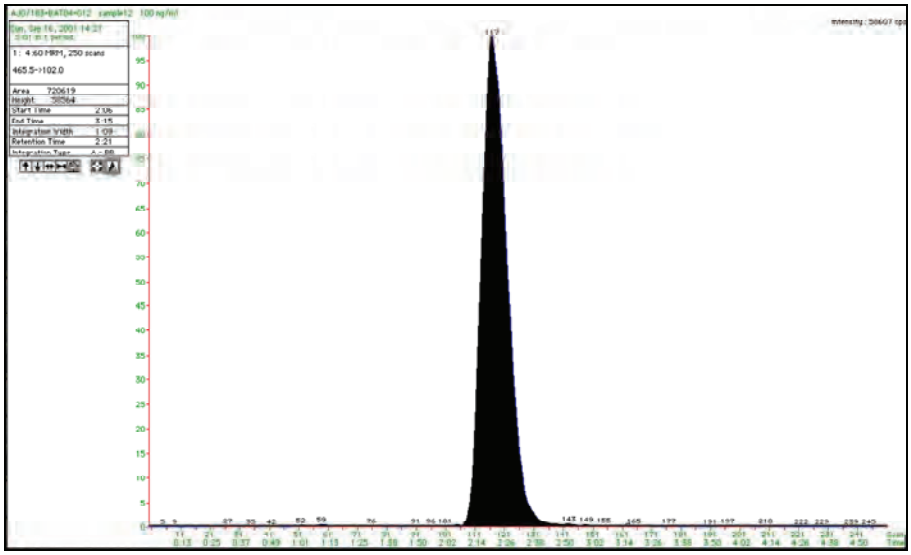
(Bioanalytical parameters – continued)

FIGURE 7

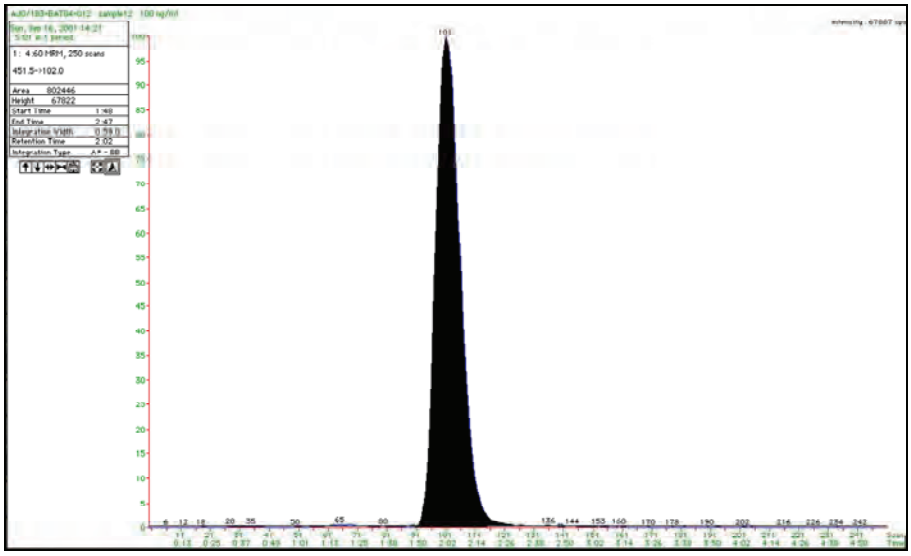
Representative chromatograms

Calibration Standard 100 ng/ml- continued

^{13}C-ANS 9801



^{13}C-ANS 9801-acid



APPENDIX 3

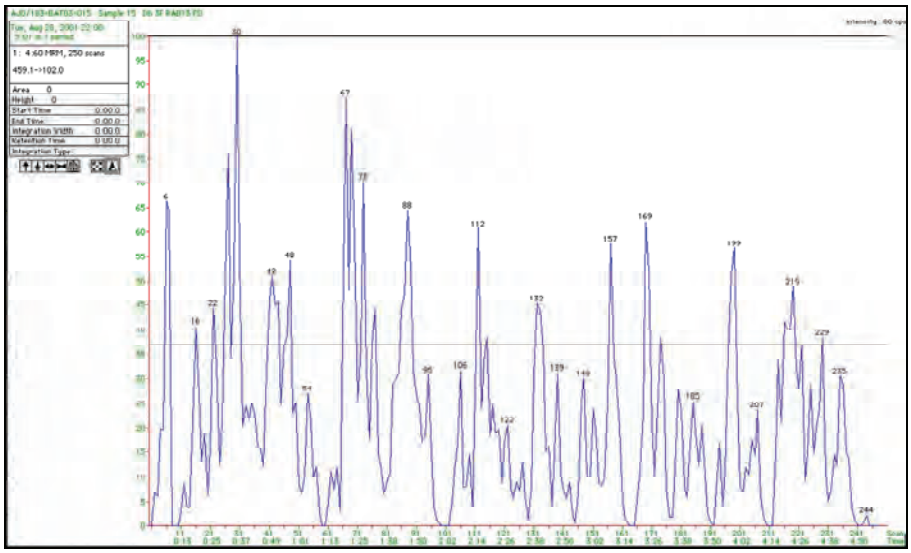
(Bioanalytical parameters - continued)

FIGURE 8

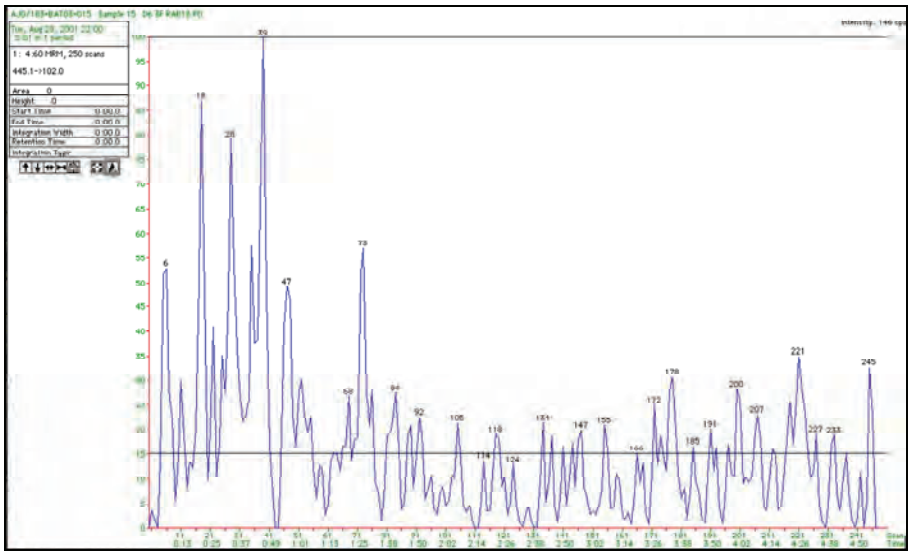
Representative chromatograms

Day 6, Group 3F, Rabbit 13, Predose

ANS 9801



ANS 9801-acid



APPENDIX 3

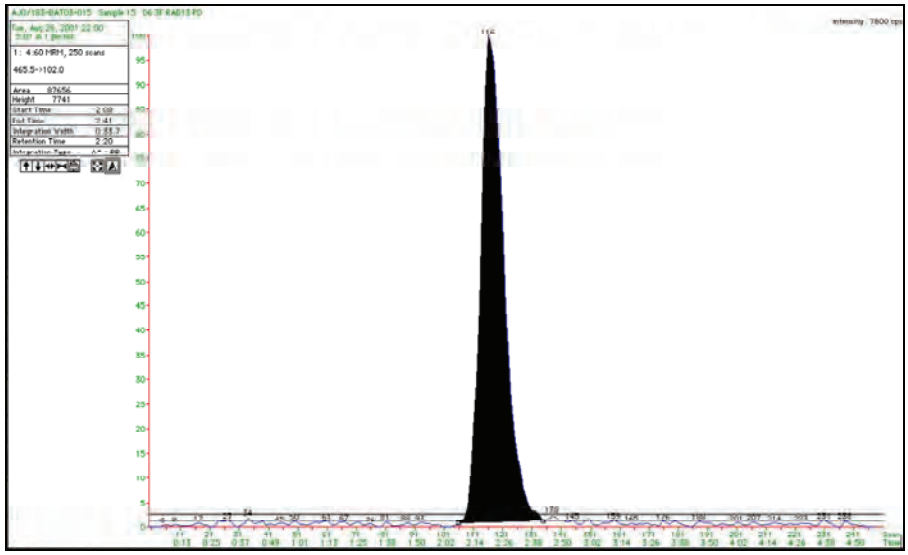
(Bioanalytical parameters - continued)

FIGURE 8

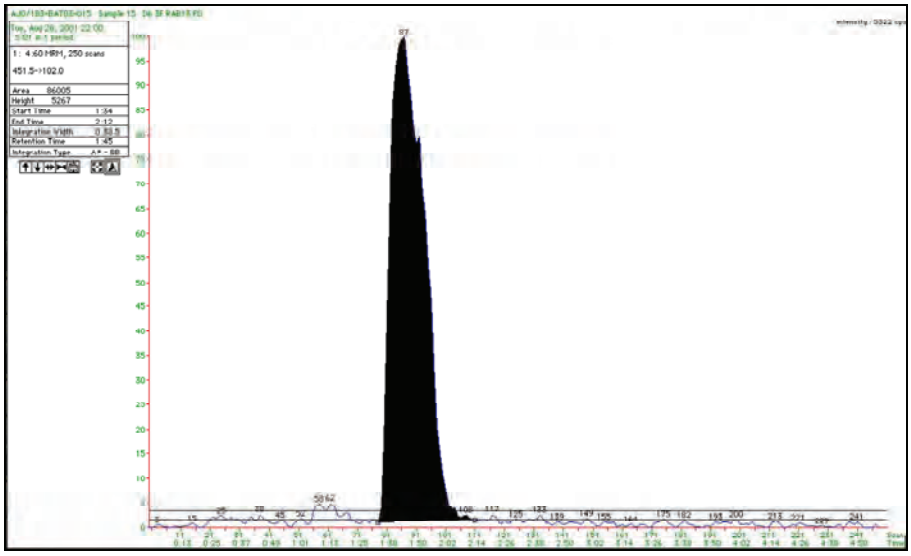
Representative chromatograms

Day 6, Group 3F, Rabbit 13, Predose

^{13}C-ANS 9801



^{13}C-ANS 9801-acid



APPENDIX 3

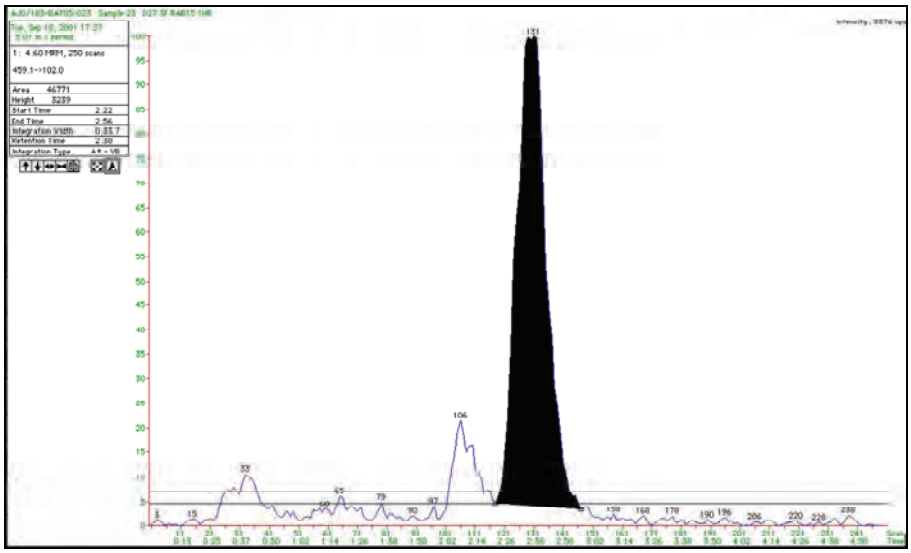
(Bioanalytical parameters - continued)

FIGURE 9

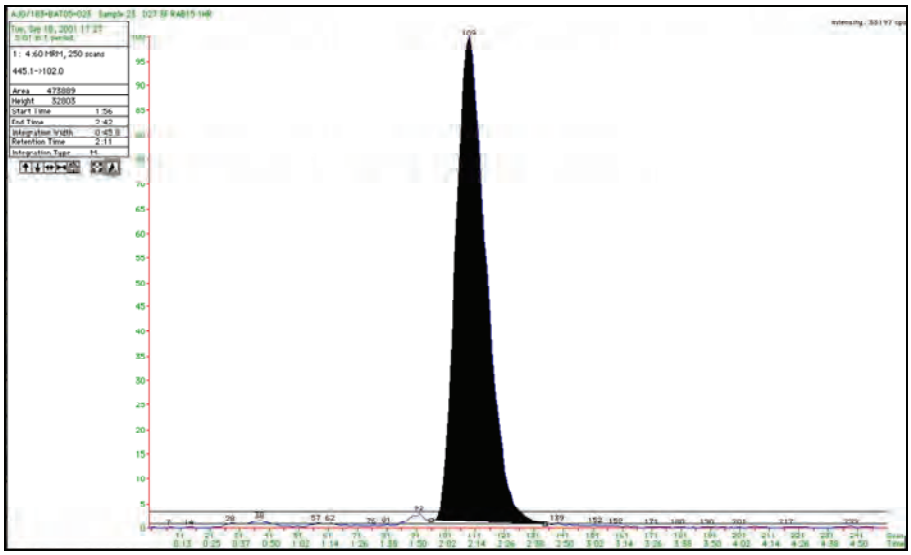
Representative chromatograms

Day 27, Group 3F, Rabbit 15, 1 hour post-dose

ANS 9801



ANS 9801-acid



APPENDIX 3

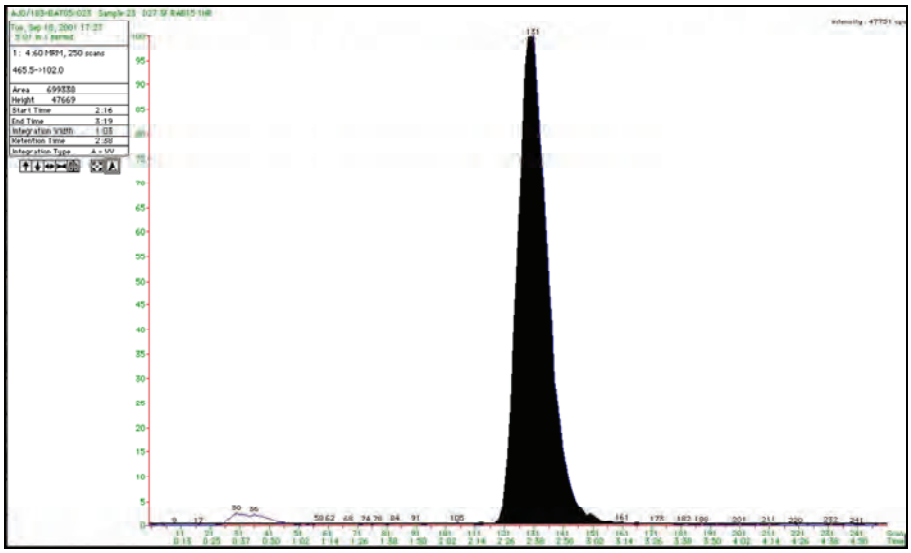
(Bioanalytical parameters - continued)

FIGURE 9

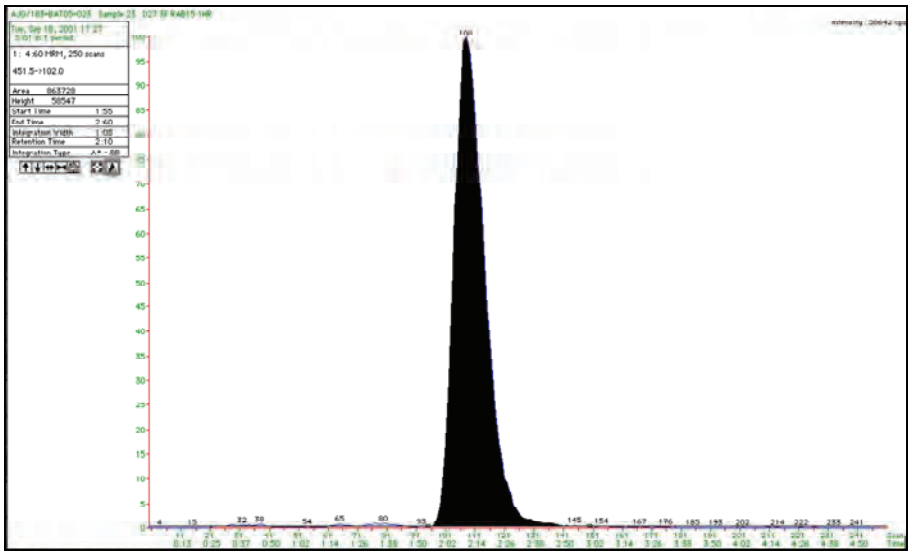
Representative chromatograms

Day 27, Group 3F, Rabbit 15, 1 hour post-dose

^{13}C-ANS 9801



^{13}C-ANS 9801-acid



APPENDIX 4

Certificates of Analyses

Certificate of analysis of ANS 9801 (Batch 00530)

DATE: 2001/8/7

CERTIFICATE OF ANALYSIS

Item Name: ANS9801 Drug Substance

Lot Number: 000530

Test item		Specifications		Result
Description		A white to yellow powder		A yellowish white powder
Identification	IR	Same as Reference Spectrum..		Same as Reference Spectrum
Purity (HPLC)	Related substance	RRT 0.27 (Aspartame)	≦1.5%	0.34%
		Other related substance	≦1.0% each	0.35%
		Total related substance	≦4.0%	1.26%
Water		2.5~5.0%		4.11%
Content (Titration)		95%~105%		100.1%

Storage condition: ANS9801 Drug Substance should be kept in a tight container at a room temperature.

Manufacturing Date: May 15, 2000

Expiration Date: May 15, 2003

This is to certify that the information listed above is a true and accurate copy of the data obtained for the material identified.

AJINOMOTO CO., INC.

Tomoya Yamamoto

Tomoya Yamamoto
Deputy General Manager
Development Research Laboratories
Pharmaceutical Laboratories

APPENDIX 4

(Certificates of Analyses – continued)

Certificate of analysis of ANS 9801-acid (AOK-0007)

DATE: Jul. 3, 2000

ANALYTICAL CHARACTERIZATION

Item Name: ANS9801 Acid Reference Standard

Lot Number: AOK-0007

Test Item		Result
Description		A white powder
Identification	^{1}H-NMR	
	^{13}C-NMR	
	Mass spectrum	
Purity(Area)	HPLC	100%(Other peaks were not detected)
Water	K.F.	2.37%

Storage condition: ANS9801 Acid Reference Standard should be kept in a tight cotainer at room temperature.

Manufacturing Date: June 14, 2000

This is to certify that the information listed above is a true and accurate copy of the data obtained the material identified.

AJINOMOTO CO.,INC.

Tadashi Takemoto

Tadashi Takemoto
Deputy General Manager
Sweetener Laboratories
Product Development Department
Aminoscience Laboratories

APPENDIX 4

(Certificates of Analyses – continued)

^{13}C-ANS 9801 (Batch number LFE/13C-ANS/82)

LFE/13C-ANS/82

Huntingdon Life Sciences
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^{13}C-ANS9801

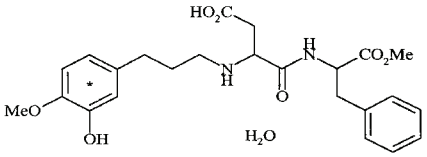
Certificate of Analysis

(re-issued 21 August 2002)

Compound : ^{13}C-ANS9801

Chemical Name: *N*-[*N*-[3-(3-hydroxy-4-methoxy-[U-^{13}C]phenyl)propyl]-α-asparty]]-*L*-phenylalanine 1-methyl ester

Structure:



Position of the ^{13}C isotope:	* Universally in the 3-hydroxy-4-methoxyphenyl ring
Isotope enrichment (^{13}C):	98.8%
Molecular Weight:	464.5 (anhydrous), 482.5 (monohydrate)
Molecular Formula:	$C_{24}H_{30}N_2O_7$. H_2O
Origin:	Synthesised at Huntingdon Life Sciences
HLS Batch No:	LFE/13C-ANS/82
Analysis:	
Melting point:	97.8-99.2°C (capillary tube)
HPLC analysis:	^{13}C-ANS9801 had the same retention time (18 minutes) as reference standard ANS9801 (lot no. 000223). No other peaks were detected in the UV chromatogram (210 nm). An Intersil ODS2 column was used with a mobile phase of phosphate buffer (pH 2.8) : acetonitrile (75 : 25, v/v) for 20 minutes followed by a gradient to a ratio of phosphate buffer (pH 2.8) : acetonitrile (50 : 50, v/v) at 50 minutes.

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APPENDIX 4

(Certificates of Analyses – continued)

(^{13}C-ANS 9801 (Batch number LFE/13C-ANS/82) – continued)

LFE/13C-ANS/82

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Mass spectra:	LC-MS of the reference standard ANS-9801 (lot no. 000530) and the ^{13}C-ANS9801 showed the two compounds had identical retention times and the fragmentation patterns (positive ion MS/MS analysis) were consistent with the proposed structures of the compounds. No impurities were observed in the ^{13}C-ANS9801 sample. The % ^{13}C incorporation in the sample was calculated as 98.83%.
Storage:	In the dark at 4°C
Expiry date:	The storage stability of ^{13}C-ANS9801 is unknown. It is recommended that the purity should be checked by LC-MS prior to use.
Analysis date:	20 February 2001

APPENDIX 4

(Certificates of Analyses – continued)

(^{13}C-ANS 9801 (Batch number LFE/13C-ANS/82) – continued)

LFE/13C-ANS/82

Huntingdon Life Sciences
Working for a better future

Personnel:

Analysis performed by:
21 August 2002
Dr L F Elsom
Scientific Manager
Environmental Metabolism and Chemistry

Approved by:
21 August 2002
Dr B C Mayo
Head of large Animal & Plant Metabolism
Environmental Metabolism and Chemistry

Quality Assurance:
21 August 2002
Tracy Scarfe
Group Manager
Department of Quality Assurance

APPENDIX 4

(Certificates of Analyses – continued)

^{13}C-ANS 9801-acid (Batch number LFE/13C-ACID/83)

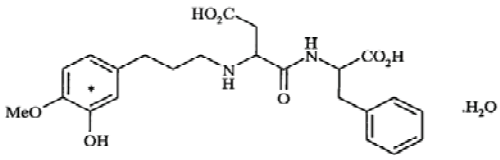
LFE/13C-ACID/83

Huntingdon Life Sciences
Working for a better future

^{13}C-ANS9801-ACID

Certificate of Analysis

(re-issued 21 August 2002)

Compound:	^{13}C-ANS9801-Acid
Chemical Name:	*N*-[*N*-[3-(3-hydroxy-4-methoxy-[U-^{13}C]phenyl)propyl]-α-aspartyl]-*L*-phenylalanine
Structure:	
Position of the ^{13}C isotope:	* Universally in the 3-hydroxy-4-methoxyphenyl ring
Isotopic enrichment (^{13}C):	98.9%
Molecular Weight:	450.5 (anhydrous), 468.5 (monohydrate)
Molecular Formula:	$C_{23}H_{28}N_2O_7 . H_2O$
Origin:	Synthesised at Huntingdon Life Sciences
HLS Batch No:	LFE/13C-ACID/83
Analysis:	
HPLC analysis:	^{13}C-ANS9801-acid had the same retention time (10 minutes) as reference standard ANS-9801-acid (lot no. AOK-0007). An Intersil ODS2 column was used with a mobile phase of phosphate buffer (pH 2.8) : acetonitrile (75 : 25, v/v) for 20 minutes followed by a gradient to a ratio of phosphate buffer (pH 2.8) : acetonitrile (50 : 50, v/v) at 50 minutes.
TLC analysis:	^{13}C-ANS9801-acid had the same R_f of 0.20 as reference standard ANS-9801-acid (lot no. AOK-0007). No impurities

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APPENDIX 4

(Certificates of Analyses – continued)

(^{13}C-ANS 9801-acid (Batch number LFE/13C-ACID/83)- continued)

LFE/13C-ACID/83

Huntingdon Life Sciences
Working for a better future

	were detected by UV or with exposure of the plate to iodine vapour.
Mass spectra:	LC-MS of the reference standard ANS-9801-acid (lot no. AOK-0007) and the ^{13}C-ANS9801-acid showed the two compounds had similar retention times (7.5 minutes) and the fragmentation patterns (positive ion MS/MS analysis) were consistent with the proposed structures of the compounds. An additional minor component (0.9%) was resolved during LC-MS of the ^{13}C-ANS9801-acid. The spectrum of this component is consistent with the fragmentation pattern and proposed structure of ^{13}C-ANS9801-acid. This minor component is therefore an isomer of the ^{13}C-ANS9801-acid. The % ^{13}C incorporation in the sample was calculated as 98.9%.
Storage:	In the dark at 4°C
Expiry date:	The storage stability of ^{13}C-ANS9801-acid is unknown. It is recommended that the purity should be checked by LC-MS prior to use.
Analysis date:	22 February 2001

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APPENDIX 4

(Certificates of Analyses – continued)

(^{13}C-ANS 9801-acid (Batch number LFE/13C-ACID/83)- continued)

LFE/13C-ACID/83

Huntingdon Life Sciences
Working for a better future

Personnel:

Analysis performed by: 21 August 2002
Dr L F Elsom
Scientific Manager
Environmental Metabolism and Chemistry

Approved by: 21 August 2002
Dr B C Mayo
Head of large Animal & Plant Metabolism
Environmental Metabolism and Chemistry

Quality Assurance: 21 August 2002
Tracy Searle
Group Manager
Department of Quality Assurance

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Study Number : AJO/183

CONFIDENTIAL

Huntingdon Life Sciences

PROTOCOL

ANS 9801

PRELIMINARY EMBRYO-FETAL TOXICITY STUDY

IN THE RABBIT BY ORAL GAVAGE ADMINISTRATION

Sponsor

Ajinomoto Co. Inc.
15-1 Kyobashi 1-chome
Chuo-ku
Tokyo 104
JAPAN

Research Laboratory

Huntingdon Life Sciences Ltd
Woolley Road
Alconbury
Huntingdon
Cambridgeshire
PE28 4HS
ENGLAND

Total number of pages: 19

Final Protocol

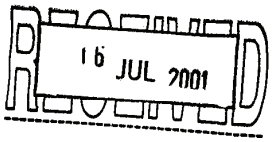
Study Number : AJO/183

Huntingdon Life Sciences

CONTACT DETAILS

Sponsor's Monitoring Scientist : Dr A. Otabe

Study Number : **AJO/183**



Huntingdon Life Sciences

PROTOCOL APPROVAL

ANS 9801

PRELIMINARY EMBRYO-FETAL TOXICITY STUDY

IN THE RABBIT BY ORAL GAVAGE ADMINISTRATION

S.M. Fulcher, B.A., F.I.A.T.
Study Director,
Huntingdon Life Sciences Ltd.

4 July 2001
Date

The signature of the Study Director confirms this protocol as the working document for the study. Any changes made subsequent to the date of the Study Director's signature will be documented in formal amendments.

P. Aughton, B.Sc., D.A.B.T., Dip.R.C.Path., C.Biol., M.I.Biol.
Management,
Huntingdon Life Sciences Ltd.

4 July '01
Date

Dr S. Tubuku
Sponsor,
Ajinomoto Co. Inc.

10 July 2001
Date

Please sign both copies of this page, retain one for your records and return one to the Study Director at Huntingdon Life Sciences.

Study Number : **AJO/183**

Huntingdon Life Sciences

ANS 9801

PRELIMINARY EMBRYO-FETAL TOXICITY STUDY IN THE RABBIT BY ORAL GAVAGE ADMINISTRATION

Enquiry Number: 21698U

Number of pages for internal distribution: 16

This working document is approved for circulation and use:

.....
Study Director

4 July 2001
.....
Date

Primary location of study

Eye Research Centre
Eye
Suffolk
IP23 7PX

Building Number: 2

All procedures to be performed at the above site unless otherwise detailed below.

Location of specific tasks

Toxicokinetics : Department of Bioanalysis, Huntingdon Research Centre, Huntingdon, PE28 4HS.

Final Protocol

Study Number : AJO/183

Huntingdon Life Sciences

CONTENTS

Study Number : AJO/183

Huntingdon Life Sciences

1. INTRODUCTION

Management of study

Study Director	:	S.M. Fulcher
Monitoring Toxicologist	:	A.M. Bottomley
In the temporary absence of the Study Director, the scientific responsibilities will be taken over by the Monitoring Toxicologist; other items of routine study management should be referred to the following person in the first instance.	:	C.R. Willoughby

Objective

Assessment of effects upon the progress and outcome of pregnancy in rabbits and to establish suitable dosages for a main embryo-fetal toxicity study.

Good Laboratory Practice

The work performed in this study will generally follow good laboratory practice principles, however, no specific study-related Quality Assurance procedures or analysis of dose form will be performed and the report may not contain all of the elements required by GLP.

Animals (Scientific Procedures) Act 1986 compliance

The in-life experimental procedures to be undertaken during the course of this study are subject to the provisions of the United Kingdom Animals (Scientific Procedures) Act 1986 (the Act). The Act, administered by the UK Home Office, regulates all scientific procedures in living animals which may cause pain, suffering, distress or lasting harm and provides for the designation of establishments where procedures may be undertaken, the licensing of trained individuals who perform the practical techniques and the issue of project licences for specified programmes of work.

This study will comply with all applicable sections of the Act and the associated Codes of Practice for the Housing and Care of Animals used in Scientific Procedures and the Humane Killing of Animals under Schedule 1 to the Act, issued under section 21 of the Act.

The number of animals used will be the minimum that is consistent with scientific integrity and regulatory acceptability, consideration having been given to the welfare of individual animals in terms of the number and extent of procedures to be carried out on each animal.

Animal model	:	Female New Zealand White (sexually mature, virgin) rabbits, background data available.
Route	:	Oral gavage, to simulate the conditions of clinical administration.

Study Number : AJO/183

Huntingdon Life Sciences

Treatment groups and dosages

Group	:	1	2	3	4
Compound	:	Control	---------- ANS 9801----------		
Dosage (mg/kg/day)	:	0	500	1000	2000

2. STUDY SCHEDULE AND STRUCTURE

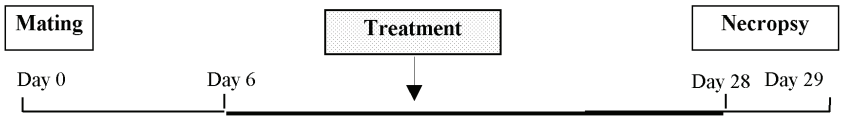
2.1. Duration of treatment

Females : Day 6 - 28 after mating.

2.2. Scheduled time plan

Mating to commence	:	23 July 2001	
Draft report to be issued	:	Mid September 2001	(estimated)

2.3. Study design



2.4. Identity of treatment groups

(to be selected from 26 animals ordered)

Group	Treatment	Dosage (mg/kg/day)* #	Number of females	Animal numbers
1	Control	0	6	1-6
2	ANS 9801	500	6	7-12
3	ANS 9801	1000	6	13-18
4	ANS 9801	2000	6	19-24

\# Expressed in terms of test substance as supplied.

* Dosage (mg/kg/day) suggested by HLS following a tolerance study in the non pregnant rabbit (ANS/166) to be confirmed by the Sponsor and documented in an amendment to the protocol.

Study Number : AJO/183

Huntingdon Life Sciences

3. TEST SUBSTANCE AND FORMULATION

In order for Huntingdon Life Sciences to comply with the Health and Safety at Work etc. Act 1974, and the Control of Substances Hazardous to Health Regulations 1999, it is a condition of undertaking the study that the Sponsor shall provide Huntingdon Life Sciences with all information available to it regarding known or potential hazards associated with the handling and use of any substance supplied by the Sponsor to Huntingdon Life Sciences. The Sponsor shall also comply with all current legislation and regulations concerning shipment of substances by road, rail, sea or air.

Such information in the form of a completed Huntingdon Life Sciences test substance data sheet must be received by Safety Management Services at Huntingdon Life Sciences before the test substance can be handled in the laboratory. At the discretion of Safety Management Services at Huntingdon Life Sciences, other documentation containing the equivalent information may be acceptable.

Information received will be used to set the Huntingdon Life Sciences Hazard Class, which determines safety precautions taken in the workplace.

Huntingdon Life Sciences Hazard Class: 2

3.1. Test substance

Sponsor's identification	:	ANS 9801
Chemical abstract name	:	*N*[N-[3-(3-hydroxy-4-methoxyohenyl)]-α aspartyl]-L-phenylalanine 1-methyl ester].
Storage conditions	:	At ambient temperature.
Sponsor's responsibilities	:	Documentation of methods of synthesis, fabrication or derivation. Stability data. Certificate of analysis.
Certificate of analysis details	:	Test substance identity. Batch number. Purity. Composition. Other appropriate characteristics. Current expiry date.

3.2. Formulation

Treatment		
Group 1, Control	:	Vehicle; 1% Methyl cellulose.
Group 2	:	ANS 9801; 100 mg/ml.
Group 3	:	ANS 9801; 200 mg/ml.
Group 4	:	ANS 9801; 400 mg/ml.
Conversion factor	:	The test substance will be used as supplied unless otherwise advised by the Sponsor. Any such requirement will be documented in an amendment to protocol.
Vehicle	:	1% Methyl cellulose.
Method of preparation	:	For each required concentration, the required amount of ANS 9801 will be weighed out and ground using a pestle and mortar. An appropriate amount of the vehicle (1% methylcellulose) is placed on top of the test substance, which is then ground again, ensuring a thorough mixing of the test material and vehicle. The suspension is then transferred to an appropriate measuring cylinder (rinsing the mortar with vehicle) and made up to volume with the required amount of vehicle. The formulation is then transferred to a beaker/jar and mixed with a magnetic stirrer for at least five minutes.
Frequency of preparation	:	Weekly.

3.3. Quality control of dosage form

Homogeneity and stability		
Determination	:	Determined by Huntingdon Life Sciences as part of the programme of work (Huntingdon Life Sciences Study Number AJO/149). No analyses will be performed as part of this preliminary study.

Study Number : AJO/183

Huntingdon Life Sciences

4. ANIMAL MANAGEMENT

4.1. Animals – supply and acclimatisation

4.1.1. Animals

Species	:	Rabbit.
Strain	:	New Zealand White.
Age ordered	:	15-18 weeks of age.
Weight range ordered	:	Approximately 2.5 – 3.5 kg.
Supplier	:	Highgate Farms accredited closed colony.
Number of females ordered	:	26.
Number of females on study	:	24

4.1.2. Acclimatisation

Duration	:	Minimum 2 weeks.
Husbandry conditions	:	Refer to Section 4.2.

4.1.3. Mating procedures‡

Method	:	Natural mating with New Zealand White bucks of established fertility.
After mating	:	Each female injected intravenously with 25 i.u. luteinising hormone.
Day 0 of gestation	:	Day of mating.

4.1.4. Allocation to treatment groups

Allocation	:	On day of mating.
Method	:	Randomised allocation, females mated on any 1 day evenly distributed amongst the groups.
Cage distribution	:	Arrangement designed to minimise environmental variables.

4.1.5. Identification

Numbering	:	Unique for each animal.
Method	:	Ear tag or ear tattoo.
Cage labels	:	Uniquely identifying the occupants.

4.1.6. Pre-commencement animal replacement

2 spare animals will be ordered to replace any individuals rejected during the acclimatisation period.

Replacement before treatment	:	All animals examined for general health and abnormalities before dosing on Day 6 after mating. Those considered unsuitable replaced.
Replacement during treatment	:	None scheduled.

4.2. Animals - housing, diet and water supply

4.2.1. Environmental control

Rabbit facility	:	Limited access.
Air supply	:	Filtered, not recirculated.
Temperature	:	Maintained within the range of 15-23ºC.
Relative humidity	:	Maintained within the range of 40-70%.

Monitored continuously or daily. Excursions outside these ranges documented in the study data.

Lighting	:	14 hours light : 10 hours dark.
Alarm systems	:	Activated on ventilation failure and when temperature limit is exceeded.
Electricity supply	:	Public supply with automatic stand-by generators.

4.2.2. Animal accommodation

One animal per cage.

Suspended cages fitted with perforated floor panels are mounted in batteries. Undertray lined with absorbent paper which is changed at least 3 times a week. Precise details of caging will be included in the final report.

4.2.3. Diet and water supply

Copies of all certificates of analysis are stored in the archives.

Diet supply

Diet name	:	Standard Rabbit Diet.
Diet type	:	Pelleted diet.
Availability	:	Non-restricted.
Certification	:	Before delivery, each batch of diet is analysed by the supplier for various nutritional components and chemical and microbiological contaminants. Supplier's analytical certificates are scrutinised and approved before any batch of diet is released for use.

This diet contains no added antibiotic or other chemotherapeutic or prophylactic agents.

Water supply

Supply	:	Public drinking water.
Regulatory agency	:	U.K. Department of the Environment.
Availability	:	Non-restricted via polyethylene bottles with sipper tubes.
Certification	:	Certificates of analysis are routinely received from the supplier.

4.2.4. Contaminants assay

It is the Sponsor's responsibility to advise Huntingdon Life Sciences of any specific contaminants likely to prejudice the outcome of the study. Analyses for such contaminants may be performed if requested by the Sponsor.

4.3. Animals - procedures

4.3.1. Administration

Route	:	Oral gavage by gastric intubation.
Treated at	:	Constant dosages in mg/kg/day. Group 2 500 mg/kg. Group 3 1000 mg/kg. Group 4 2000 mg/kg.
Volume dosage	:	5 ml/kg/day.
Individual dose volume	:	Calculated from the most recently recorded scheduled bodyweight.

Controls (Group 1)	:	Vehicle at same volume dosage as treated groups.
Frequency	:	Once daily at approximately the same time each day.
Sequence	:	By group.
Reason for selection of duration	:	The length of treatment was selected to cover embryogenesis and fetal development from the time of implantation until shortly before expected delivery date.
Reason for selection of concentrations	:	Based on the results of a tolerance study (AJO/166) in which non-pregnant females tolerated a level of 2000 mg/kg/day for 14 days. 2000 mg/kg/day was considered to be the maximum practical dose level.
Formulation	:	A daily record of the usage of formulation will be maintained based on weights. This balance is compared with the expected usage as a check of correct administration. Formulations are stirred using a magnetic stirrer before and throughout the dosing procedure, unless otherwise directed by the Sponsor.

4.3.2. Clinical signs

Animals and their cages	Visually inspected daily throughout the study for evidence of reaction to treatment or ill-health.
Deviations from normal recorded at the time in respect of	Nature and severity. Date and time of onset. Duration and progress of the observed condition.

In addition detailed observations will be performed to establish and confirm a pattern of signs according to the following minimum schedule:

Schedule	:	1. At the end of dosing all groups. 2. Approximately ½ hour after dosing. 3. Approximately 1 hour after dosing. 4. Approximately 2 hours after dosing. 5. Approximately 4 hours after dosing.

The above schedule will be amended, as necessary, in the light of signs observed.

4.3.3. Mortality

Premature sacrifice	:	Animals may be killed on humane grounds or if considered *in extremis*.
Animals found dead, killed *in extremis* or on humane grounds	:	A necropsy is performed as soon as possible. Animals found outside the normal workday will be preserved in a refrigerator (approximately 4°C) provided for this purpose.

4.3.4. Maternal bodyweight

All animals	:	Daily throughout study.

4.3.5. Food consumption

Each animal	:	Daily throughout study.

4.3.6. Water consumption

Assessed visually each day	:	Recorded over 24 hour period for Days 1, 6, 13, 20 and 27

4.4. Toxicokinetics

The sampling schedule will be as follows:

Day No.	Group	Time of sampling (hours after dosing)							
		Predose	0.5	1	2	4	8	12	24
		Animal numbers							
6 and 26	1	1-3	4-6	1-3	4-6	1-3	4-6	1-3	4-6
	2	7-9	10-12	7-9	10-12	7-9	10-12	7-9	10-12
	3	13-15	16-18	13-15	16-18	13-15	16-18	13-15	16-18
	4	19-21	22-24	19-21	22-24	19-21	22-24	19-21	22-24

Conditions	:	Without overnight deprivation of food.
Sample site	:	Central auricular artery.
Anticoagulant	:	Lithium heparin + 6 µL diethyl-p-nitrophenyl phosphate (Paraoxan) in ethanol (5 mg/ml).
Sample volume	:	1.0 ml.
Total number of samples taken	:	192 (96 on each occasion).

Separation of plasma	:	By centrifugation at approximately 4ºC. From each sample, two 200 µL aliquots of plasma will each be mixed with 20 µL 1.0M aqueous citric acid solution, snap frozen and then stored at approximately -70°C.
Storage of plasma	:	Appropriately labelled plastic tubes. Deep frozen (approximately -70°C), pending analysis by Huntingdon Life Sciences.

Samples from all treated groups will be analysed.

Bioanalysis by

Department	:	Department of Mass Spectrometry.
Site	:	Huntingdon Research Centre
Bioanalytical method	:	LC-MS/MS

Bioanalysis will be subject to the satisfactory validation of the bioanalytical method. Details are documented in a separate Methodology Validation protocol (or report if available).

Method Validation Study Number	:	AJO/170.
Total number of samples to be analysed	:	144 (288 aliquots). Control samples not analysed. One aliquot of each sample will be used for the initial analysis. The second aliquot of each sample will be held pending a possible requirement for repeat analysis. Any such requirement will be documented in the study data.
Sample analysis	:	Bioanalysis of test samples will be performed using a validated method. Appropriate quality control (QC) samples will also be used.

Each batch of samples will be analysed together with a set of calibration standards prepared freshly each day. If data exist to show that the analyte is stable in the biological matrix, calibration standards may be prepared in advance and stored deep-frozen until use.

a) QC samples will be made up in blank plasma, prepared from stock solutions of the test substance weighed out specifically for their preparation.
b) Ideally sufficient QC's will be made up prior to the start of bioanalysis to allow for use with all study samples.
c) QC's will be prepared at the following concentrations:

   i) Low (2 to 3 times limit of quantification).
   ii) Medium (30% to 60% of upper limit of quantification or approximate geometric mean of calibration range).
   iii) High (70% to 90% of upper limit of quantification).

d) All QC samples will be stored deep-frozen (approximately -80°C) together with the test samples until analysed. These will be analysed at least in duplicate at each concentration with each analytical batch.

e) Acceptance criteria for each analytical batch will be that at least 4 of the 6 QC results are within 15% (20% for the low QC) of their respective nominal values, with at least one valid result at each concentration.

Pharmacokinetic and statistical analysis appropriate to the data will be performed, and would normally include, at least, determination of maximum plasma concentration (C_{max}), time of maximum plasma concentration (T_{max}), and area under the plasma concentration time-curve (AUC).

4.5. Animals - termination

All animals will be subject to terminal investigations (Section 5).

5. NECROPSY AND HISTOLOGY

5.1. Time of necropsy

Surviving females	:	Day 29 after mating.
Females that exhibit pregnancy loss	:	Same day that abortion is detected.

5.2. Method of kill

All animals	:	Intravenous injection of pentobarbitone.
Fetuses	:	Subcutaneous injection of pentobarbitone.

5.3. Macroscopic pathology

5.3.1. Examination of animals found dead, killed *in extremis* or on humane grounds or that exhibit pregnancy loss.

Macroscopic examination of the visceral organs will be performed to identify the cause of death and abnormal tissues are retained. Uterine contents examined and numbers of corpora lutea and implantation sites recorded. Where possible fetuses will also be examined.

5.3.2. Examination of animals scheduled for necropsy

Macroscopic examination will be performed for evidence of disease or adverse reaction to treatment and abnormal tissues retained.

The number corpora lutea in each ovary will be counted and the reproductive tract, complete with ovaries, will be dissected out. The weight of the gravid uterus will be recorded. The following will be recorded:

Each ovary/uterine horn	:	Number of: Corpora lutea. Implantation sites. Resorption sites (assessed as early: no evidence of embryonic or fetal tissue, or late: evidence of fetal development present). Fetuses (live and dead). Fetuses and placentae dissected from the uterus and weighed individually.
All fetuses	:	External examination. Internal examination of neck and thoracic and abdominal cavities. Sex recorded. Individual identification within litter. One third of the fetuses in each litter will be decapitated and the heads initially fixed in Bouin's fluid. Remaining fetuses and torsos will be eviscerated and fixed in Industrial Methylated Spirits (74° o.p.).
Apparently non-pregnant animals	:	Status confirmed by Salewski staining technique for presence of implantation sites.

Photographs may be prepared showing representative treatment-related macroscopic abnormalities

5.4. Histology and light microscopy (Optional)

Histological processing and microscopic examination of retained tissues will only be performed, and documented in an amendment to the protocol, if requested by the Sponsor.

5.5. Photomicrography (Optional)

Images	:	Illustration of fetal findings or major lesions after consultation with the Sponsor; taken by a Pathologist.
Report	:	Full micrography report if required.

Study Number : AJO/183

Huntingdon Life Sciences

6. DATA TREATMENT

6.1. Data processing

Summary data presented as mean with standard deviation (SD).

Bodyweight : Group mean values and SD calculated from individual data for Days 0, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26 and 28 after mating. Weight changes presented graphically from Day 6 after mating.

Food consumption : Group mean values and SD will be calculated daily.

Water consumption : Group mean values and SD will be calculated for specified days.

Reproductive tract, Day 29 after mating : Group mean values and SD calculated:
Corpora lutea
Implantations
Resorptions (early, late, total)
Viable young (male, female, total).

Pre-implantation loss :

$$\frac{\text{Number corpora lutea - Number implantations}}{\text{Number corpora lutea}} \times 100$$

Post-implantation loss :

$$\frac{\text{Number implantations - Number live fetuses}}{\text{Number implantations}} \times 100$$

Fetal weights : Group mean values and SD calculated for male, female and overall from -

$$\frac{\text{Total of individual litter mean fetal weights}}{\text{Number of litters}}$$

Placental weights : Group mean values and SD calculated from -

$$\frac{\text{Total of individual litter placental weights}}{\text{Number of litters}}$$

6.2. Statistical analysis

The small sample size precludes meaningful statistical evaluation.

7. REPORTING

Study progress : Periodic verbal and written updates on study progress will be provided by the Study Director. A routine synopsis report will be sent at termination of the in-life phase.

Draft final report : For review by the Sponsor.

Authorised final report : After approval from the Sponsor.

Routinely reports are supplied on A4 paper. The following numbers of reports are supplied:

Type of report	Printing	Number of copies	
		Bound	Unbound
Draft report	Double-sided	0	2
Authorised final	Double-sided	1	0
	Single-sided	0	1
Photographic report (if any)	Single-sided	1	0

Any additions or corrections to an authorised final report will be documented as a formal addendum/amendment to the final report.

In the absence of ongoing communications, Huntingdon Life Sciences reserves the right to finalise, sign and issue the final report from this study six months after issue of the draft. In such an event, all materials will be transferred to the archive. Any subsequent requests for modifications, corrections or additions to the final report will be the subject of a formal report amendment (or new study, as appropriate) and will be subject to additional cost.

8. QUALITY ASSURANCE AND ARCHIVING PROCEDURES

8.1. Quality assurance

No formal study-based Quality Assurance procedures will be performed on this study. These may be included if requested by the Sponsor.

8.2. Archiving

All raw data, samples and specimens arising from the performance of this study will remain the property of the Sponsor.

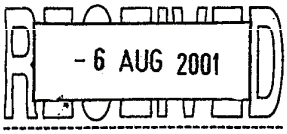
Types of sample and specimen which are unsuitable, by reason of instability, for long term retention and archiving may be disposed of after the periods stated in Huntingdon Life Sciences Standard Operating Procedures.

All other samples and specimens and all raw data will be retained by Huntingdon Life Sciences in its archive for a period of five years from the date on which the Study Director signs the final report. After such time, the Sponsor will be contacted and his advice sought on the return, disposal or further retention of the materials. If requested, Huntingdon Life Sciences will continue to retain the materials subject to a reasonable fee being agreed with the Sponsor.

Huntingdon Life Sciences will retain the Quality Assurance records relevant to this study and a copy of the final report in its archive indefinitely.

Study Number : **AJO/183**
Protocol Amendment Number : **1**

Huntingdon Life Sciences



ANS 9801

PRELIMINARY EMBRYO-FETAL TOXICITY STUDY

IN THE RABBIT BY ORAL GAVAGE ADMINISTRATION

Total number of pages: 6

Number of pages for internal distribution: 6

Study Director : **S.M. Fulcher, B.A., F.I.A.T.**

The signature of the Study Director authorises the implementation of this amendment to protocol. In this amendment, deleted statements are struck through and new statements are underlined. Any changes to the study design after the date of this authorising signature will be documented in a further formal amendment.

FIRST AMENDMENT APPROVAL

For Huntingdon Life Sciences Ltd

Authorised by: _____ Date: 24 July 2001
(Study Director)

For the Sponsor

Approved by: _____ Date: 30 July 2001

Study Number : **AJO/183**
Protocol Amendment Number : **1**

Huntingdon Life Sciences

ANS 9801

PRELIMINARY EMBRYO-FETAL TOXICITY STUDY

IN THE RABBIT BY ORAL GAVAGE ADMINISTRATION

Reason for amendment	:	Section 2.2: Correction of commencement of mating date.
	:	Section 2.4: Correction of study number for tolerance study in the non pregnant rabbit, previously run at HLS.
	:	Section 4.1.2: Correction to typographical error, acclimatisation period is approximately 2 weeks.
	:	Section 4.3.2: Addition of pre-dose observation. Addition of "or as late as possible in the working day" to 4 hour post dose observation, to cover weekend working hours.
	:	Section 4.3.6: Day 6 amended to Day 8 and Day 27 amended to Day 26 to avoid recording of water consumption on bleed days.
	:	Section 4.4: Day 26 toxicokinetic bleed amended to Day 27 to avoid recording of water consumption on bleed days and bleeding procedure occurring at weekend.

Study Number : AJO/183
Protocol Amendment Number : 1

Huntingdon Life Sciences

Amendments

2. STUDY SCHEDULE AND STRUCTURE

2.2 Scheduled time plan

Mating to commence	:	~~23~~ 24 July 2001	
Draft report to be issued	:	Mid September 2001	(estimated)

2.4. Identity of treatment groups

(to be selected from 26 animals ordered)

Group	Treatment	Dosage (mg/kg/day)* #	Number of females	Animal numbers
1	Control	0	6	1-6
2	ANS 9801	500	6	7-12
3	ANS 9801	1000	6	13-18
4	ANS 9801	2000	6	19-24

\# Expressed in terms of test substance as supplied.
* Dosage (mg/kg/day) suggested by HLS following a tolerance study in the non pregnant rabbit (~~ANS~~ AJO/166) to be confirmed by the Sponsor and documented in an amendment to the protocol.

4. ANIMAL MANAGEMENT

4.1. Animals – supply and acclimatisation

4.1.2 Acclimatisation

Duration	:	~~Minimum~~ Approximately 2 weeks.
Husbandry conditions	:	Refer to Section 4.2.

Study Number : **AJO/183**
Protocol Amendment Number : **1**

Huntingdon Life Sciences

4.3. Animals - procedures

4.3.2 Clinical signs

Animals and their cages	Visually inspected daily throughout the study for evidence of reaction to treatment or ill-health.
Deviations from normal recorded at the time in respect of	Nature and severity. Date and time of onset. Duration and progress of the observed condition.

In addition detailed observations will be performed to establish and confirm a pattern of signs according to the following minimum schedule:

Schedule : 1. Pre-dose observations.
~~1~~ 2. At the end of dosing all groups.
~~2~~ 3. Approximately ½ hour after dosing.
~~3~~ 4. Approximately 1 hour after dosing.
~~4~~ 5. Approximately 2 hours after dosing.
~~5~~ 6. Approximately 4 hours after dosing or as late as possible in the working day.

The above schedule will be amended, as necessary, in the light of signs observed.

4.3.6. Water consumption

Assessed visually each day : Recorded over 24 hour period for Days 1, ~~6~~ 8, 13, 20 and ~~27~~ 26

4.4 Toxicokinetics

The sampling schedule will be as follows:

Day No.	Group	Time of sampling (hours after dosing)							
		Predose	0.5	1	2	4	8	12	24
		Animal numbers							
~~6 and~~ ~~26~~ 27	~~1~~	1-3	4-6	1-3	4-6	1-3	4-6	1-3	4-6
	2	7-9	10-12	7-9	10-12	7-9	10-12	7-9	10-12
	3	13-15	16-18	13-15	16-18	13-15	16-18	13-15	16-18
	4	19-21	22-24	19-21	22-24	19-21	22-24	19-21	22-24

Conditions : Without overnight deprivation of food.

Study Number : AJO/183
Protocol Amendment Number : 1

Huntingdon Life Sciences

Sample site	:	Central auricular artery.
Anticoagulant	:	Lithium heparin + 6 µL diethyl-p-nitrophenyl phosphate (Paraoxan) in ethanol (5 mg/ml).
Sample volume	:	1.0 ml.
Total number of samples taken	:	192 (96 on each occasion).
Separation of plasma	:	By centrifugation at approximately 4ºC. From each sample, two 200 µL aliquots of plasma will each be mixed with 20 µL 1.0M aqueous citric acid solution, snap frozen and then stored at approximately -70°C.
Storage of plasma	:	Appropriately labelled plastic tubes. Deep frozen (approximately -70°C), pending analysis by Huntingdon Life Sciences.

Samples from all treated groups will be analysed.

Bioanalysis by

Department	:	Department of Mass Spectrometry.
Site	:	Huntingdon Research Centre
Bioanalytical method	:	LC-MS/MS

Bioanalysis will be subject to the satisfactory validation of the bioanalytical method. Details are documented in a separate Methodology Validation protocol (or report if available).

Method Validation Study Number	:	AJO/170.
Total number of samples to be analysed	:	144 (288 aliquots). Control samples not analysed. One aliquot of each sample will be used for the initial analysis. The second aliquot of each sample will be held pending a possible requirement for repeat analysis. Any such requirement will be documented in the study data.
Sample analysis	:	Bioanalysis of test samples will be performed using a validated method. Appropriate quality control (QC) samples will also be used.

Each batch of samples will be analysed together with a set of calibration standards prepared freshly each day. If data exist to show that the analyte is stable in the biological matrix, calibration standards may be prepared in advance and stored deep-frozen until use.

a) QC samples will be made up in blank plasma, prepared from stock solutions of the test substance weighed out specifically for their preparation.
b) Ideally sufficient QC's will be made up prior to the start of bioanalysis to allow for use with all study samples.
c) QC's will be prepared at the following concentrations:

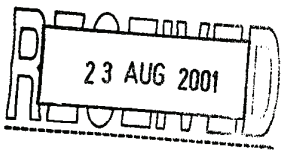
   i) Low (2 to 3 times limit of quantification).
   ii) Medium (30% to 60% of upper limit of quantification or approximate geometric mean of calibration range).
   iii) High (70% to 90% of upper limit of quantification).

d) All QC samples will be stored deep-frozen (approximately -80°C) together with the test samples until analysed. These will be analysed at least in duplicate at each concentration with each analytical batch.
e) Acceptance criteria for each analytical batch will be that at least 4 of the 6 QC results are within 15% (20% for the low QC) of their respective nominal values, with at least one valid result at each concentration.

Pharmacokinetic and statistical analysis appropriate to the data will be performed, and would normally include, at least, determination of maximum plasma concentration (C_{max}), time of maximum plasma concentration (T_{max}), and area under the plasma concentration time-curve (AUC).

Study Number : **AJO/183**
Protocol Amendment Number : **2**

Huntingdon Life Sciences



ANS 9801

PRELIMINARY EMBRYO-FETAL TOXICITY STUDY

IN THE RABBIT BY ORAL GAVAGE ADMINISTRATION

Total number of pages: 2

Number of pages for internal distribution: 2

Study Director : S.M. Fulcher, B.A., F.I.A.T.

The signature of the Study Director authorises the implementation of this amendment to protocol. In this amendment, deleted statements are struck through and new statements are underlined. Any changes to the study design after the date of this authorising signature will be documented in a further formal amendment.

SECOND AMENDMENT APPROVAL

For Huntingdon Life Sciences Ltd

Authorised by: _____ Date: 7 August 2001
(Study Director)

For the Sponsor

Approved by: _____ Date: 17 Aug. 2001

Study Number : **AJO/183**
Protocol Amendment Number : **2**

Huntingdon Life Sciences

ANS 9801

PRELIMINARY EMBRYO-FETAL TOXICITY STUDY

IN THE RABBIT BY ORAL GAVAGE ADMINISTRATION

Reason for amendment : Section 2.2: Correction of commencement of mating date.
Correction of issue of draft report date.

Amendments

2. STUDY SCHEDULE AND STRUCTURE

2.2 Scheduled time plan

Mating to commence	:	~~24 July~~ 7 August 2001
Draft report to be issued	:	~~Mid September 2001~~ 12 October 2001 (estimated)

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE IN ACCORDANCE WITH DIRECTIVE 88/320 EEC

LABORATORY	TEST TYPE
Huntingdon Life Sciences **Eye Research Centre** **Eye** **Suffolk** **IP23 7PX**	**Analytical Chemistry** **Clinical Chemistry** **Ecosystems** **Environmental Fate** **Environmental Toxicity** **Mutagenicity** **Phys/Chem Testing** **Toxicology**

DATE OF INSPECTION

29th January 2001

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of UK GLP Compliance Programme.

At the time of the inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

Roger J. Alexander
3/4/01

Dr. Roger G. Alexander
Head, UK GLP Monitoring Authority

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE IN ACCORDANCE WITH DIRECTIVE 88/320 EEC

LABORATORY	TEST TYPE
Huntingdon Life Sciences **Huntingdon Research Centre** **Wooley Road** **Alconbury** **Huntingdon** **Cambs.** **PE28 4HS**	**Analytical Chemistry** **Clinical Chemistry** **Ecosystems** **Environmental Fate** **Environmental Toxicity** **Phys/Chem Testing** **Toxicology**

DATE OF INSPECTION

15th January 2001

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of UK GLP Compliance Programme.

At the time of the inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

Roger G. Alexander
3/4/01

Dr. Roger G. Alexander
Head, UK GLP Monitoring Authority