# NARASIN

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## IDENTITY

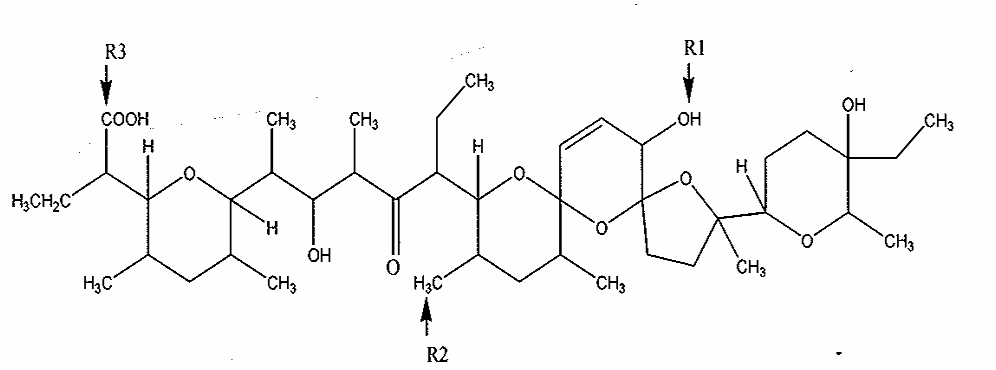
**International Non-proprietary names (INN):** Narasin

**Synonyms:** (4s)-4-methylsalinomycin, Narasin A, Monteban®, Naravin®

**International Union of Pure and Applied Chemistry (IUPAC) Names:** α-ethyl-6-[5-[2-(5 ethyltetrahydro-5-hydroxy-6-methyl-2H-pyran-2-yl)-15-hydroxy-2, 10, 12-trimethyl-1, 6, 8-trioxadispiro [4.1.5.3] pentadec-13-en-9-yl]-2-hydroxy-1, 3-dimethyl-4oxoheptyl] tetrahydro-3,5-dimethyl-2H-pyran-2-acetic acid.

**Chemical Abstract Service (CAS) Number:** 55134-13-9

**Structural formula of main components:**




| Structural variants of Narasin | R1 | R2 | R3 |
|---|---|---|---|
| **A** | OH | $CH_3$ | COOH |
| **B** | =O | $CH_3$ | COOH |
| **D** | OH | $C_2H_5$ | COOH |
| **I** | OH | $CH_3$ | $COOCH_3$ |

**Molecular formula of Narasin A:** $C_{43}H_{72}O_{11}$ (C 67.41%, H 9.49%, O 23.01%)

**Molecular weight:** 765.02

## OTHER INFORMATION ON IDENTITY AND PROPERTIES

**Pure active ingredient:** Narasin A

**Appearance:** Crystal from acetone-water

**Melting point:** 98 - 100°C (crystal from acetone-water)
158 - 160ºC (crystalline narasin sodium salt)

**Solubility:** Soluble in alcohol, acetone, DMF, DMSO, benzene, chloroform, ethyl acetate. Insoluble in water.

## RESIDUES IN FOOD AND THEIR EVALUATION

### Conditions of use

Narasin belongs to the polyether monocarboxylic acid class of ionophores produced by *Streptomyces aureofaciens* strain NRRL 8092. Narasin is composed of 96% Narasin A, 1% Narasin B, 2% narasin D and 1% narasin I. The biological activity of narasin is based on its ability to form lipid soluble and dynamically reversible complexes with cations, preferably monovalent cations such as alkaline $K^+$, $Na^+$ and $Rb^+$: Narasin functions as a carrier of these ions, mediating an electrically neutral exchange-diffusion type of ion transport across the membranes. The resultant changes in transmembrane ion gradients and electrical potentials produce critical effects on cellular function and metabolism of coccidia. Narasin is effective against sporozoites and early and late asexual stages of coccidia in broilers caused by *Eimeria acervulina, E. brunetti, E. maxima, E. mivati, E. necatrix* and *E. tenella*. Narasin also is used for prevention of necrotic enteritis in broiler chicken.

The antimicrobial spectrum of activity of narasin is limited mainly to Gram-positive bacteria including *Enterococcus* spp.*, Staphylococcus* spp., and *Clostridium perfringens*. Narasin is not used in human medicine and it is not classified as a critically important antibiotic for human use by expert meetings convened by WHO (WHO, 2007). It has, however been classified by OIE (OIE, 2007) as an important antibiotic for veterinary medicine for control of coccidiosis.

### Dosage

Narasin has been approved for use in chickens for fattening at dose of 60-80 mg of active substance/kg of complete feed (54-72 gram per 2000 lb ton).

## PHARMACOKINETICS AND METABOLISM

Because the principal effect of narasin is on the microflora of the intestinal tract (including coccidia); few conventional pharmacokinetic studies have been performed. Studies in both target and laboratory animals indicate that narasin is rapidly metabolised in liver and eliminated in faeces within a few days.

### Pharmacokinetics in Laboratory Animals

Rats

A non-GLP compliant metabolism study was performed in rats in order to evaluate the absorption and excretion of narasin (Manthey, 1977a). A single oral dose of 2.3 mg of $^{14}$C-labelled narasin with a specific activity of 0.596 µCi/mg was used. Rats were maintained in metabolism cages designed to separate the urine from the faeces. Food and water were provided *ad libitum.* Total radioactivity recovered in the urine and faeces was 75% of the administered dose at 52 hrs post-dosing. Only 1.1% of the total excreted radioactivity was found in the urine and the remainder was in the faeces (98.9%). In a study with three young mature rats surgically prepared for bile collection, approximately 15% of the dose was recovered in the bile samples indicating that a substantial portion of the $^{14}$C narasin dose was absorbed and processed through the hepatic system.

### Pharmacokinetics in Food Animals

Chickens

Three non-GLP compliant studies were evaluated.

In the first study (Peippo, et al., 2005), 30 males and 30 females broilers chickens (Ross 508-hybrid) were fed an un-medicated starter broiler ration from one-day -old until two weeks of age. For the duration of the study, chickens were fed a grower ration that contained 0, 3.5 or 70 mg narasin/kg of feed. Throughout the study, water and feed were supplied *ad libitum*. During the withdrawal period, chickens were again fed a non-medicated grower feed. At slaughter, samples of muscle were removed and blood was collected into heparin tubes. All the samples were stored at -20°C until analysed. Concentrations of narasin in the plasma and muscle of chickens were determined by time-resolved fluoroimmunoassay and results are shown in Table 1.

**Table 1: Concentrations of narasin in plasma and muscle of broilers treated with 3.5 or 70 mg narasin/kg feed.**

| Feeding conditions | Bird number | Narasin concentration | | |
|---|---|---|---|---|
| | | Plasma (µg/L) | Leg muscle (µg/kg) | Breast muscle (µg/kg) |
| Feed containing 0 mg/kg of narasin | 1 | ND | ND | ND |
| | 2 | ND | ND | ND |
| | 3 | ND | ND | ND |
| | 4 | ND | ND | ND |
| Feed containing 3.5 mg narasin /kg; no withdrawal period | 1 | 1.6 | 0.7 | 1.2 |
| | 2 | 1.8 | 0.6 | 0.7 |
| | 3 | 4.2 | 1.7 | 0.6 |
| | 4 | 3.4 | 1.6 | 1.3 |
| Feed containing 70 mg narasin /kg; no withdrawal period | 1 | 39.8 | 2.4 | 2.1 |
| | 2 | 59.3 | 4.2 | 2.3 |
| | 3 | 70.2 | 6.2 | 4.5 |
| Feed containing 70 mg narasin /kg; 3 day withdrawal period | 1 | ND | ND | ND |
| | 2 | ND | ND | ND |
| | 3 | ND | ND | ND |
| Feed containing 70 mg narasin /kg; 5 day withdrawal period. | 1 | ND | ND | ND |
| | 2 | ND | ND | ND |
| | 3 | ND | ND | ND |

ND: Not detected
Limit of detection (LOD): 0.6 µg/kg
Limit of quantification (LOQ): 1.8 µg/kg

The narasin concentration in plasma was related to the concentration of narasin in the medicated feed. Plasma concentrations increased nearly 20 times when the narasin concentrations in feed were increased twenty times. In contrast, narasin concentrations in the muscle of chickens that were medicated with 70 mg narasin/kg feed increased only two-fold compared to chickens that were fed with 3.5 mg narasin/kg feed. While higher concentrations of narasin in medicated feed result in proportionally higher residue concentrations in plasma and muscle, the increase is not always a dose proportional increase in tissues. Narasin was not detected in plasma and muscle at the 3- and 5-day withdrawal periods indicating that narasin disappears rapidly from poultry tissues after the administration of the compound.

In the second study (Catherman, et al., 1991), 30 mature chicken hens (Single Comb White Leghorn) were housed individually in metabolism cages. $^{14}C$-labelled narasin was injected via cardiac puncture (0.7 µCi in 100µl of 85% dimethyl sulfoxide and 15% saline as a vehicle). Blood samples were taken from 8 chickens at different hours post-injection from 0.5 to 18 h. Excreta were collected daily from individual hens. Groups of 6 chickens were killed by cervical dislocation on days 1, 7, 14 and 28 post-injection and were necropsied to recover liver, kidney, heart, ovary, fat, skin, bile and muscle.

Approximately 80% of the dose cleared from the plasma before the first blood sample was taken (0.5 h) and at 24 hours post-injection only trace amounts remained. Liver, heart, fat, skin and ovarian tissues contained traces of radioactivity 1 day post-injection. Muscle and kidney contained no detectable concentrations of $^{14}C$ on day 1. All organ tissues cleared the radiolabel by day 7 and no detectable radioactivity was present thereafter. In excreta, the highest amount of $^{14}C$ was founded on day 1 (49% of dose) and by day 13 there was no detectable radioactivity. Approximately 93.6% of the administered dose was eliminated in the excreta. The radioactivity is reported in Table 2.

**Table 2: Activity and concentration of $^{14}C$ in excreta of chickens. [1, 2]**

| CHICKENS | | | |
|---|---|---|---|
| Day | n[3] | (% of dose) | (µg/kg)[4] |
| 1 | 24 | 48.9 ± 3.4 | 725 ± 60 |
| 2 | 18 | 19.9 ± 2.8 | 371 ± 60 |
| 3 | 18 | 13.1 ± 2.1 | 163 ± 21 |
| 4 | 18 | 6.6 ± 1.1 | 66 ± 11 |
| 5 | 18 | 1.7 ± 0.4 | 26 ± 13 |
| 6 | 18 | 0.6 ± 0.6 | 4 ± 1 |
| 7 | 18 | 0.2 ± 0.1 | 2 ± 1 |
| 8 | 12 | 0.6 ± 0.2 | 5 ± 2 |
| 9 | 12 | 1.2 ± 0.5 | 12 ± 6 |
| 10 | 12 | 0.2 ± 0.1 | 2 ± 0.7 |
| 11 | 12 | 0.4 ± 0.1 | 3 ± 0.7 |
| 12 | 12 | 0.2 ± 1 | 1 ± 0.7 |
| 13 | 12 | 0 | 0 |
| 14 | 12 | 0 | 0 |

[1] Chicken was dosed with 0.7 µCi as narasin. Recovered radioactivity was assumed to remain associated with the narasin molecule.
Total excreta samples were collected daily.
[2] Values are ± S.E
[3] n= number of samples, each from an individual chicken.
[4] Narasin equivalents, micrograms per kilogram of excreta.

In the third study (Manthey, 1977a), 4 broilers chickens approximately eight weeks old and preconditioned to narasin at 80 mg/kg in feed, were each given a single oral capsule dose of $^{14}C$-labelled narasin. Excreta were collected from each chicken daily (24 hour samples) and analysed for radiochemical content. More than 85 % of the dose was recovered within 48 hours.

Quail

In a non-GLP compliant study (Catherman, et al., 1991), 60 Japanese quail hens were randomly assigned to five groups of 12 hens each. The quails were injected with $^{14}C$-labelled narasin via cardiac puncture (0.113 µCi in 50µl of 85% dimethyl sulfoxide and 15% saline as a vehicle). Blood samples were taken from 8 quails at different hours post-injection. Groups of 12 quails were killed by cervical dislocation on days 1, 7, 14 and 28 days post-injection and were necropsied to recover the liver, kidney, heart, ovary, fat, skin, bile and muscle. Excreta were collected daily (1 only at day 14). Approximately 92% of the dose cleared plasma before the first blood sample was taken (0.5 h) and at 24 hours post-injection only trace amounts remained. No detectable concentrations could be found at 7 days post-injection.

In the excreta, 68.2 % of $^{14}C$ was recovered on day 1 and 75% within 72 hours. Liver, heart, fat and ovarian tissues contained traces of radioactivity on 1 day post-injection. Muscle and kidney contained

no detectable concentrations of $^{14}C$ on day 1. All organ tissues cleared the radiolabel by day 7 and no detectable concentrations of $^{14}C$ narasin were present thereafter.

Cattle

A GLP compliant study (Manthey, et al., 1984a) was conducted to investigate the rate, route and quantitative nature of the excretion of $^{14}C$-labelled narasin from 2 Hereford heifers. The cattle were acclimated to confinement in metabolism cages for approximately one week prior to dosing. To assure separation of urine from the faeces, animals were fitted with indwelling urethral catheters. Each heifer was given a single dose of $^{14}C$ narasin (about 11.0 μCi of radioactivity was placed singly in a gelatine capsule). Following dosing, the urine and faeces were collected quantitatively daily at about 24-hours intervals. A total of 93.4% and 80.1% of the administered radioactivity was recovered; up to 98% in the faeces and less than 0.5% in the urine. The radioactivity in the faeces was excreted within 4 days of dosing.

In a non-GLP compliant study (Manthey, at al., 1982), Hereford feedlot cattle (6 steers and 3 heifers) were dosed orally with an amount of $^{14}C$-labelled narasin corresponding to narasin usage at about 19.8 mg/kg. The cattle were confined in metabolism cages and dosed each morning and evening for 3, 5 and 7 days. At 12 hours following the last dosing, the animals were slaughtered and muscle, back fat, kidney and liver were collected.

Liver contained the highest concentration of residues corresponding to 0.92, 0.74 and 0.84 mg narasin/kg equivalents from cattle dosed for 3, 5 and 7 days, respectively. Through one-way analysis of variance of the means, the liver residue values were not statistically different, indicating that steady-state equilibrium of total tissue residue was established within 3 days of dosing. In contrast, little more than trace concentrations of residues were found in the other tissues (0.006 and 0.03 mg/kg equivalents all days). In these tissues, the mean residue concentrations were not statistically different from all animals at all dosing periods. The residues did not reflect the duration of dosing, or differences in animal size or sex.

Pigs

Two GLP compliant studies were conducted to evaluate the pharmacokinetics of narasin in pigs.

In the first study (Sweeney, et. al., 1995), three groups of 4 pigs were fed $^{14}C$-labelled narasin rations for 7 days at 30 mg/kg with zero withdrawal (treatment 1), 30 mg/kg with a three-day withdrawal (treatment 2) and 45 mg/kg with zero withdrawal (treatment 3). Urine and faeces were collected daily throughout the study. At slaughter, samples of liver, kidney, muscle, back fat, skin and bile were collected.

Radioactivity measurements showed that 3-5% of the recovered radioactivity was found in urine and 95-97% in the faeces. Liver was the edible tissue with the highest amount of residue for all treatment groups. The other tissues contained relatively little residue. The amounts of radioactive residues in the edible tissues are shown in Table 3.

**Table 3: Summary of radiolabelled residues (mg/kg-equivalents) in the edible tissues of $^{14}C$ - narasin-treated pigs.**

| Treatment Group 1- 30 mg/kg- zero withdrawal | | | | | |
|---|---|---|---|---|---|
| Animal Number | Liver | Kidney | Muscle | Skin | Fat |
| 71 | 0.63 | 0.04 | ND | 0.02 | 0.05 |
| 75 | 0.99 | 0.05 | ND | 0.07 | 0.11 |
| 77 | 0.60 | 0.04 | ND | 0.02 | 0.03 |
| 79 | 0.79 | 0.04 | ND | 0.04 | 0.06 |
| Mean | 0.75 | 0.04 | ND | 0.04 | 0.06 |
| | | | | | |
| Treatment Group 2- 30 mg/kg- 3 days withdrawal | | | | | |
| Animal Number | Liver | Kidney | Muscle | Skin | Fat |
| 64 | 0.18 | 0.01 | ND | ND | 0.02 |
| 69 | 0.18 | 0.02 | ND | 0.02 | 0.02 |
| 76 | 0.16 | 0.01 | ND | ND | 0.01 |
| 78 | 0.14 | 0.01 | ND | 0.01 | 0.02 |
| Mean | 0.17 | 0.01 | ND | 0.02 | 0.02 |
| | | | | | |
| Treatment Group 3- 45 mg/kg- zero withdrawal | | | | | |
| Animal Number | Liver | Kidney | Muscle | Skin | Fat |
| 63 | 1.19 | 0.09 | 0.02 | 0.04 | 0.09 |
| 70 | 1.80 | 0.09 | 0.01 | 0.04 | 0.08 |
| 72 | 0.96 | 0.07 | ND | 0.05 | 0.07 |
| 80 | 1.96 | 0.09 | 0.02 | 0.07 | 0.15 |
| Mean | 1.48 | 0.09 | 0.02 | 0.05 | 0.10 |

ND: No detectable residue based on mean of the control tissue cpm value plus three times the standard deviation.

In a second study (Donoho, et al., 1988), six crossbred pigs (4 males and 2 females) were fed a ration containing $^{14}C$-labelled narasin at a concentration equivalent to 37.5 mg/kg. The pigs were placed into separate metabolism crates. Three pigs (2 males and 1 female) were fed for 9 days and a similar group was fed for 5 days. All of the animals were killed at 12 hours after the last dose.
The mean liver radioactivity concentration at zero withdrawal was 0.51 mg/kg equivalents for the pigs dosed for 5 days and 0.55 mg/kg for the pigs dosed for 9 days. There was no statistical difference between the two groups indicating that 5 days was an adequate period to establish steady-state concentrations. In one male pig, urine and faeces were collected daily. Approximately 6-8% of the administered dose was recovered in urine and 92-94% was recovered in the faeces.

**Metabolism in Laboratory Animals**

Rats

In a GLP compliant study (Sweeney and Kennington, 1994), 10 male and 10 female Fischer strain 344 rats were given daily oral (gavage) doses of 5 mg narasin/kg bw for 5 days. Urine and faeces were collected daily from all animals and extracted for metabolite profiling. Narasin metabolites in the extract were identified by high performance liquid chromatography/ion spray-mass spectrometry (HPLC/ISP-MS).

In the faeces, four structural isomers of tri-hydroxy narasin A and four di-hydroxy narasin A were identified. Four peaks were identified as tri-hydroxy narasin B and four as di-hydroxynarasin B. Using high performance liquid chromatography/ion spray-mass spectrometry (HPLC/ISP/MS), the exact

position of hydroxylation could not be determined in this study. These metabolites demonstrate that the narasin metabolic pathways in the rat include hydroxylation and oxidation.

In another GLP compliant study (Manthey and Goebel, 1986), 6 mature rats, 3 males and 3 females, were placed in individual metabolism cages. The rats were dosed by gavage for 5 consecutive days. Each dose was 1 mL of the acacia $^{14}C$ -narasin suspension, which corresponded to a dose of about 3.3 mg $^{14}C$-narasin/kg bw. Faeces were collected daily during the dosing period. About four hours after the last dose, the rats were killed, necropsied and the livers were collected immediately. In this study, $^{14}C$-narasin was metabolized to more than twenty metabolites and the pattern in faeces and liver was qualitatively similar.

Dogs

In a GLP compliant study (Manthey and Goebel, 1986), a mature male dog, weighing 11.8 kg, was placed in a metabolism cage and acclimated for 4 days prior to dosing. This study was conducted to make a comparison between cattle, rat and dog. The animal was dosed by oral gavage for 4 consecutive days; one-half of the allotted dose was given in the morning and the other half at mid-day. The dose was 2.0 mg $^{14}C$-narasin/kg bw. The faeces were collected each day and stored in a freezer during the dosing period. Urine was not collected. About 4 hours after the last dose, the dog was euthanized by injection of sodium phenobarbital. The liver was excised immediately, chopped and frozen. The study demonstrated that $^{14}C$-narasin is metabolized to more than 20 metabolites by those species. No single metabolite accounts for a large proportion of the total. The pattern of narasin metabolites in faeces and liver is qualitatively similar among the three species, although there are quantitative differences. The primary metabolic pathway appears to be oxidation (hydroxylation) of the narasin at various sites on the polyether rings. The metabolites that have been identified are mono- di- or tri-hydroxy narasin derivates.

**Metabolism in Food Producing Animals**

Cattle

As noted above, a GLP compliant study (Manthey and Goebel, 1986) was conducted to compare the metabolism of $^{14}C$ narasin in orally dosed cattle (target animal), dog and rats. This study indicated that the pattern of narasin metabolites in faeces and liver was qualitatively similar among the three species, although there were quantitative differences. Liver is the only edible tissue in cattle that contains appreciable concentrations of residue. The most abundant metabolite in cattle liver is NM-12, a mono-hydroxy narasin, which accounts for approximately 15% of the liver radioactivity. Metabolite NM-13 (di-hydroxy narasin) is relatively abundant in cattle faeces.

Pigs

In a GLP compliant study (Sweeney, et. al., 1995), three groups of 4 pigs were fed $^{14}C$-narasin rations for 7 days containing 30 mg narasin/kg with zero withdrawal (treatment 1), 30 mg narasin/kg with three day withdrawal (treatment 2) and 45 mg narasin/kg with zero withdrawal (treatment 3). Pigs were individually housed in metabolism cages. Urine and faeces were collected from each animal daily throughout the study. Pigs were slaughtered by captive bolt and exsanguination and samples of liver, kidney, skin, muscle, back fat and bile were collected.

Narasin metabolites were characterized using high performance liquid chromatography/electrospray-mass spectrometry/liquid scintillation counting (LC/EPS-MS/LSC). Liver contained the greatest amount of residue in all treatment groups; other tissues containing relatively low residues. The mean concentration of radioactivity in all tissues was greater in pigs fed 45 mg narasin/kg than those fed 30 mg narasin/kg at zero withdrawal. In the group that was fed with 30 mg/kg after a three-day withdrawal, the total residues in each tissue had depleted to one-fourth in liver and kidney, to one one-half in skin and to one third in fat of the concentrations at zero withdrawal, respectively. A number of hydroxylated metabolites of narasin and narasin B were identified in the liver, bile and faeces at zero withdrawal. The total ion chromatograms, radiochromatograms and mass spectra for bile and faeces

are similar to those seen for liver. Five hydroxylated metabolites were identified as being common to both faeces and liver. The metabolic profile of narasin in liver, bile and faeces is summarized in Tables 4, 5 and 6. These data show that narasin is extensively metabolized by pigs and hydroxylation is the main metabolic pathway in liver, bile and faeces.

**Table 4: Metabolites in pig liver from TIC (Total Ion Chromatogram) and radiochromatogram in pigs fed 30 mg narasin/kg with zero withdrawal.**

| Metabolite ID | TICpeak | RC* peak | Ammoniated/Sodiated molecular ion | % Injected Radioactivity | Proposed structure* |
|---|---|---|---|---|---|
| N-1 | A | 1 | 828/833, 830/835 | 4.3 | OH3B,OH3 |
| N-3 | B | 2 | 828/833, 830/835 | 5.2 | OH3B,OH3 |
| N-4, N-5 | C | 3 | 812/817 | 10.3 | OH2B |
| | D | 4 | 812/817 | 4.4 | OH2B |
| N7 | E | 5 | 814/819 | 3.8 | OH2 |
| | F | 6 | 814/819 | 3.0 | OH2 |
| | G | 7 | 814/819 | 6.2 | OH2 |
| | H | 8 | 814/819 | 2.2 | OH2 |
| | I | 9 | 798/803 | 1.2 | OH |
| | | | % Total Injected Radioactivity | 40.6 | |

**Table 5: Metabolites in pig bile from TIC (Total Ion Chromatogram) and radiochromatogram in pigs fed 30 mg narasin/kg with zero withdrawal.**

| TICpeak | RC* peak | Ammoniated/Sodiated Molecular ion | % Injected Radioactivity | Proposed structure* |
|---|---|---|---|---|
| A | 1 | 830/835 | 4.1 | OH3 |
| B | 2 | 830/835, 828/833 | 14.4 | OH3, OH3B |
| C | 3 | 830/835 | 4.6 | OH3 |
| D | 4 | 814/819,812/817 | 13.6 | OH2, OH2B |
| E | 5 | 812/817 | 9.4 | OH2B |
| F | 6 | 812/817 | 3.4 | OH2B |
| G | 7 | 814/819 | 2.7 | OH2 |
| H | 8 | 814/819 | 5.5 | OH2 |
| I | 9 | 814/819 | 5.1 | OH2 |
| % Total Injected Radioactivity | | | 62.8 | |

**Table 6: Metabolites in pig faeces from TIC (Total Ion Chromatogram) and radiochromatogram in pigs fed with 30 mg narasin/kg with zero withdrawal.**

| Metabolite ID | TIC peak | RC* peak | Ammoniated/Sodiated molecular ion | % Injected Radioactivity | Proposed structure* |
|---|---|---|---|---|---|
| N-2 | A | 1 | 830/835 | 3.3 | OH3 |
| N-3 | B | 2 | 828/833 | 4.4 | OH3B |
| | C | 3 | 812/817 | 4.8 | OH2B |
| N-4 | D | 4 | 812/817 | 20.9 | OH2B |
| N-5 | E | 5 | 812/817 | 4.1 | OH2B |
| N-6 | F | 6 | 814/819 | 3.8 | OH2 |
| | G | 7 | 814/819 | 1.3 | OH2 |
| | H | 8 | 812/817 | 6.5 | OH2B |
| N-7 | I | 9 | 814/819 | 5.3 | OH2 |
| | J | 10 | 814/819 | 2.1 | OH2 |
| | K | 11 | 814/819 | 1.6 | OH2 |
| | L | 12 | 814/819 | 2.2 | OH2 |
| | M | 13 | 782/787 | 3.8 | Narasin |
| | N | 14 | 780/785 | 0.3 | Narasin B |
| % Total Injected Radioactivity | | | | 64.6 | |

* OH2 = di-hydroxynarasin, OH3 = tri-hydroxynarasin

Chickens

Two GLP compliant studies were conducted to evaluate the metabolism of narasin in chickens. In the first study (Holmstrom, et al., 2002), the metabolic fate of $^{14}C$ -narasin in the edible tissues and excreta of 20 broiler chickens was studied at practical zero withdrawal (6 hour) following 5 consecutive days of treatment with medicated feed provided *ad libitum*. The feed contained a nominal 80 mg narasin/kg (71.1 mg narasin/kg measured as narasin A). The animals were housed in individual stainless steel cages in a temperature controlled environment. Six control broilers received un-medicated feed. Excreta were collected daily beginning one day prior to dosing until the day of slaughter. Livers were collected at necropsy.

Extensive metabolism of narasin A was noted in the liver with oxidative hydroxylation as the primary pathway of metabolism. The predominant metabolites are di-hydroxylated and tri-hydroxylated narasin A, representing 42% of total radioactivity injected. Narasin A metabolites identified in excreta included hydroxylated, di-hydroxylated and tri-hydroxylated narasin A and di and tri-hydroxylated analogs of an oxidized form due to ketone formation. These metabolites represented 88.9% of total radioactivity injected. Identified metabolites and their respective calculated concentrations in liver and excreta are shown in Table 7.

**Table 7: Quantification of metabolites in liver and excreta by radiochromatograms collected concomitantly using mass spectrometry.**

| Liver | | | |
|---|---|---|---|
| Metabolite ID | Proposed metabolite structure | Percent of total Radioactivity Injected | Estimated Concentration[1] in Liver, mg/kg |
| NL3 | Trihydroxynarasin A | 16 | 0.04 |
| NL1,NL2 | Dihydroxynarasin A | 8 | 0.02 |
| NL4 | Trihydroxynarasin A | 18 | 0.05 |
| Total | | 42 | 0.12 |
| Excreta | | | |
| Metabolite ID | Proposed metabolite structure | Percent of total Radioactivity Injected | Estimated Concentration[1] in Liver, mg/kg |
| NE6 | Trihydroxynarasin A | 6.9 | 4.9 |
| NE7 | Trihydroxynarasin A | 19.6 | 13.9 |
| NE8 | Trihydroxynarasin A | 6.2 | 4.4 |
| NE10 | 9-Keto-trihydroxynarasin A | 6.2 | 4.4 |
| NE3 | Dihydroxynarasin A | 2.9 | 2.1 |
| NE1<br>NE9<br>NE12 | Hydroxynarasin A<br>Trihydroxynarasin A<br>Trihydroxynarasin A | 3.7 | 2.6 |
| NE4<br>NE11 | Dihydroxynarasin A<br>9-Keto-trihydroxynarasin A | 34.4 | 24.3 |
| E5 | 9-Keto-dihydroxynarasin A | 8.6 | 6.1 |
| NE2 | Narasin A<br>Hydroxynarasin A | 0.4 | 0.3 |
| Total | | 88.9 | 62.8 |

[1]Calculated by multiplying mean residue concentration by fraction of total radioactivity injected.

In the second study (Sweeney, et al., 1994), 5 chickens were fed rations containing 50 mg $^{14}C$ narasin/kg feed. Excreta were collected from each pen beginning 1 day before initiation of the study and continuing until the end of treatment. After 5 days, the chickens were slaughtered and samples of liver, kidney, muscle, fat and skin/fat were collected and assayed for total radioactivity by solubilisation and liquid scintillation counting.

Liver was the tissue with highest concentration of extractable radioactivity (61%) but individual metabolites could not be identified because of the low amount of radioactivity in the liver. Kidney and muscle had a mean concentration ≤0.05 mg/kg and fat, skin/fat ≤ 0.12 mg/kg. At least fifteen metabolites and parent narasin were identified from the excreta. These metabolites were predominately di and tri-hydroxylated narasin A and di and tri-hydroxylated narasin B. The distribution and relative magnitude of radioactivity from liver and excreta were similar, suggesting that excreta metabolites are the same as those found in liver. The results and indicated molecular ions for each metabolite in excreta are shown in Table 8.

**Table 8: Narasin metabolites characterized in excreta. Peak determined from overlay of TIC* on the radiochromatogram.**

| TIC peak number | Radio chromatogram peak number | Ammoniated/Sodiated Molecular Ion | % Total radioactivity | Proposed structure |
|---|---|---|---|---|
| | 1 | | 1.0 | Tetrahydroxynarasin |
| A | 2 | 846/851 | 2.4 | Trihydroxynarasin |
| B | 3 | 830/835 | 13.4 | Trihydroxynarasin |
| C | 3 | 830/835 | ** | Trihydroxynarasin B |
| D | 4 | 828/833 | 6.9 | Trihydroxynarasin B |
| E | 4 | 828/833 | ** | Trihydroxynarasin B |
| F | 4 | 828/833 | ** | Trihydroxynarasin B |
| G | 5 | 828/833 | 3.0 | Trihydroxynarasin B |
| H | 5 | 828/833 | ** | Trihydroxynarasin B |
| I | 6 | 812/817 | 6.2 | Dihydroxynarasin B |
| J | 7 | 814/819 | 4.4 | Dihydroxynarasin |
| K | 7 | 814/819 | ** | Dihydroxynarasin |
| L | 8 | 812/817 | 1.9 | Dihydroxynarasin B |
| M | 9 | 814/819-812/817 | 1.88 | Dihydroxynarasin /Dihydroxynarasin B |
| N | 10 | 814/819-828/833 | 1.86 | Dihydroxynarasin /Trihydroxynarasin B |
| O | 11 | 828/833 | 0.48 | Trihydroxynarasin B |
| % of Total Radioactivity | | | 43.46 | |

In a non-GLP compliant study, six metabolites of narasin were isolated from excreta of chickens that were fed a ration containing 100 mg $^{14}C$ narasin/kg. Four metabolites were tentatively identified as di-hydroxynarasin and two as tri-hydroxynarasin. The six metabolites were assayed for antimicrobial activity against *Bacillus subtilis* in a standard narasin TLC bioautographic assay system. These metabolites were 20 times less active than narasin (Manthey and Goebel, 1982).

## TISSUE RESIDUE DEPLETION STUDIES

### Radiolabelled Residue Depletion Studies

Cattle

In a GLP compliant study (Manthey, et al., 1984b), Hereford feedlot cattle, 6 steers and 3 heifers, naïve to narasin and weighing between 185-220 kg, were used as test and control animals. The cattle were confined in individual metabolism cages located in a temperature-controlled barn. Each animal received a single capsule with $^{14}C$ narasin equivalent to 13 mg/kg feed administered orally using a bolus gun. The animals were dosed morning and evening for 5 consecutive days. At each of the withdrawal times of zero (12 hours after the final capsule dose), 1 and 3 days, cattle were killed. Samples of liver, kidney and back fat were collected immediately for radiochemical analysis. The mean net radiochemical residues were calculated as mg narasin/kg equivalents.

Liver contained the highest concentrations of radioactivity corresponding to 0.49, 0.23 and 0.05 mg narasin /kg equivalents at the withdrawal times of zero, 1 and 3 days, respectively. Less than 5% of the liver radioactivity corresponded to parent narasin. Muscle, fat and kidney contained less than 0.02 mg narasin/kg equivalents at zero withdrawal. Results are provided in Table 9.

**Table 9: Mean net[a] radioactivity in tissues of cattle following oral dosing with $^{14}$C-narasin at a concentration equivalent to a 13 mg/kg ration.**

| Tissue radioactivity as mg/kg Narasin equivalents | | | | | | |
|---|---|---|---|---|---|---|
| Animal Number | Sex | Days Withdrawal | Liver | Kidney | Back Fat | Muscle |
| 915 | F | 0 | 0.49 | 0.01 | 0.02 | 0.003 |
| 871 | F | 0 | 0.39 | 0.01 | 0.01 | 0.006 |
| 862 | M | 0 | 0.60 | 0.1 | 0.02 | NNR[b] |
| Mean | | | 0.49 | 0.01 | 0.02 | |
| | | | | | | |
| 916 | F | 1 | 0.19 | 0.002 | 0.003 | 0.002 |
| 876 | M | 1 | 0.28 | 0.002 | 0.009 | 0.002 |
| 867 | M | 1 | 0.23 | 0.004 | 0.002 | NNR[b] |
| Mean | | | 0.23 | 0.003 | 0.005 | |
| | | | | | | |
| 914 | F | 3 | 0.04 | NNR[b] | 0.001 | NNR[b] |
| 905 | M | 3 | 0.05 | NNR[b] | NNR[b] | 0.004 |
| 861 | M | 3 | 0.07 | NNR[b] | 0.001 | 0.002 |
| Mean | | | 0.05 | | | |

a) Net mg/kg equivalent to: net dpm/g ÷779 dpm/µg

b) No net residue. Negative net values were derived for these samples

Pigs

In a GLP compliant study (Donoho, et al., 1988), pigs (male and female) weighing approximately 22 kg, were fed a ration containing $^{14}$C-narasin equivalent to 37.5 mg/kg for 5 days. Half of the daily dose was given in the morning and the other half in the evening. Groups of 3 pigs were killed at 0 (12 hours after the last dose), 24, 48 or 72 hours withdrawal time. Muscle, liver, kidney, skin and fat were assayed for total radioactivity. Total radioactivity in liver for 0, 24, 48 and 72 h withdrawal were 0.51, 0.44, 0.26 and 0.18 mg/kg-equivalents, respectively. Muscle and kidney contained no radioactivity at zero withdrawal and fat contained less than 0.05 mg/kg equivalents of narasin. Other withdrawal times were not assayed because zero residues were of no practical significance. The results are showed in Table 10.

**Table 10: Radioactivity concentrations of narasin in tissues of pigs.**

| Net Radioactivity (mg narasin equivalents/kg) | | | | | | |
|---|---|---|---|---|---|---|
| Animal Number and Sex | Dosing Period | Withdrawal Time (hours) | Liver | Muscle | Kidney | Fat |
| H136859-M | 5 day | Zero | 0.37 | NDR | NDR | NDR |
| H136890-M | 5 day | Zero | 0.42 | NDR | NDR | NDR |
| H136896-F | 5 day | Zero | 0.74 | NDR | NDR | 0.04 |
| | | mean ± s.d | 0.51 ± 0.2 | | | |
| | | | | | | |
| H131886-M | 5 day | 24 hrs. | 0.49 | - | - | - |
| H131882-M | 5 day | 24 hrs. | 0.43 | - | - | - |
| H131876-F | 5 day | 24 hrs. | 0.40 | - | - | - |
| | | mean ± s.d | 0.44 ± 0.04 | | | |
| | | | | | | |
| H131880-M | 5 day | 48 hrs. | 0.28 | - | - | - |
| H131881-M | 5 day | 48 hrs. | 0.24 | - | - | - |
| H131884-F | 5 day | 48 hrs. | 0.27 | - | - | - |
| | | mean ± s.d | 0.26 ± 0.02 | | | |
| | | | | | | |
| H131878-M | 5 day | 72 hrs. | 0.18 | - | - | - |
| H131879-M | 5 day | 72 hrs. | 0.19 | - | - | - |
| H131885-F | 5 day | 72 hrs. | 0.18 | - | - | - |
| | | mean ± s.d | 0.18 ± 0.01 | | | |

NDR: No detectable residue.

Chickens/Turkeys

In a non-GLP compliant study (Manthey, et al., 1983), male and female chickens were grown from one day of age using a nominal 80 mg narasin/kg ration. At about eight weeks of age the birds were dosed with 80 mg $^{14}$C-narasin (1.35 or 1.01 µCi/mg)/kg ration *ad libitum* for 5 days and then slaughtered at zero, 1 and 3 days of withdrawal. Muscle, liver, kidney, skin and fat were assayed for total radioactivity. Radioactivity concentration in tissues was presented as mg narasin/kg-equivalents. Liver contained the highest $^{14}$C-residues and muscle contained the lowest. At 3 days withdrawal all residues were below 0.025 mg/kg equivalents with the exception of liver, which was approximately 0.07 mg/kg equivalents.

In a non GLP compliant study (Manthey, 1977b), 12 broilers chickens were grown for eight weeks on feed that contained 80 mg narasin/kg. The chickens then received capsule doses of $^{14}$C-narasin, each of which contained 4.6mg (0.297 µCi/mg $^{14}$C-narasin) orally morning and evening for two and one-half days. During this period and the withdrawal periods, the chickens were maintained on non-medicated feed. Withdrawal times were zero (four hours after the last dose), 1, 2, 3, 5 and 7 days. One male and one female were sacrificed at each withdrawal time and muscle, liver, kidney and fat tissues and skin were collected.

At zero withdrawal time, radiochemical residues were found in all tissues except muscle. Liver contained the highest residue concentration, which represented 0.50 mg narasin/kg equivalents. After two days withdrawal, the concentration declined by 93% and no residue exceeded 0.04 µg narasin/kg equivalents. The tissue residues declined progressively throughout the withdrawal period to negligible concentrations. The results are shown in Table 11.

**Table 11: Net radiochemical residues as mg narasin/kg-equivalents in tissues of chickens treated orally with $^{14}$C-narasin.**

| Withdrawal Time (days) | Sex | Muscle | Liver | Kidney | Fat | Skin |
|---|---|---|---|---|---|---|
| Zero | M | ND[1] | 0.01 | ND | 0.04 | 0.05 |
| | F | ND | 0.50 | 0.11 | 0.22 | 0.17 |
| 1 | M | ND | 0.13 | ND | ND | 0.06 |
| | F | ND | 0.12 | ND | 0.13 | 0.08 |
| 2 | M | [2] | ND | ND | ND | 0.02 |
| | F | | 0.04 | ND | ND | 0.04 |
| 3 | M | [2] | ND | ND | ND | 0.03 |
| | F | | 0.04 | ND | ND | 0.02 |
| 5 | M | [2] | ND | ND | ND | 0.02 |
| | F | | ND | ND | ND | 0.00 |

[1] No net residue exceeded the 95% upper confidence limit of control mean
[2] Not assayed

In a GLP compliant study (Manthey, et al., 1981), broiler chickens approximately seven week of age were dosed for 5 days with a broiler ration containing 100 mg $^{14}$C narasin/kg. At each of five withdrawal intervals, zero, 1, 2, 3 and 5 days, three birds (two male and one female) were sacrificed. Muscle liver, kidney, fat, muscle and skin samples were taken from each chicken. Total radioactivity was determined by combustion analysis and scintillation counting and the mean net radiochemical residues were calculated as mg narasin/kg equivalents.

The zero withdrawal time values of narasin in mg/kg equivalents were: liver, 0.45; fat, 0.21; skin, 0.14; kidney, 0.14; and muscle, 0.02. Following withdrawal of medication, the radiochemical residues declined sharply in all tissues. After a 1 day withdrawal, the residue concentrations had declined by more than 50 percent and all tissues except liver were below 0.1 mg narasin/kg equivalents. A summary of these data is given in Table 12.

**Table 12: Net[1] radioactivity as mg narasin/kg equivalents in chickens fed 100 mg $^{14}$C narasin/kg feed.**

| Withdrawal Time (days) | Mean values mg/kg equivalents (n=3) | | | | |
|---|---|---|---|---|---|
| | Muscle | Liver | Kidney | Fat | Skin |
| Zero | 0.02 | 0.45 | 0.14 | 0.21 | 0.14 |
| 1 | 0.01 | 0.18 | 0.05 | 0.06 | 0.06 |
| 2 | 0.01 | 0.12 | 0.03 | 0.02 | 0.02 |
| 3 | 0.01 | 0.13 | 0.03 | 0.01 | 0.03 |
| 3 | 0.01 | 0.10 | 0.02 | 0.01 | 0.03 |

[1] Net mg/kg-equivalent to: (gross dpm/g – control dpm/g) ÷ 932 dpm/µg

**Residue Depletion Studies with Unlabelled Drug**

*Residues in Tissues*

Cattle

In a non-GLP compliant study (Potter and Cooley, 1975), the residue pattern over different withdrawal times (0, 24, 48 and 120 h) was determined using a TLC-bio-autographic method. Eighteen Hereford cattle were allotted by weight to four treatment groups. The animals were fed 150 mg narasin/head/day (65 mg narasin/kg) for 140 days. At the time of slaughter, representative samples of muscle, fat, liver

and kidney were collected. The results showed concentrations less than 5 µg/kg of narasin in the muscle tissue at zero withdrawal and no residues were found at subsequent sampling times. Residues were found in the fat and liver up to 48 hrs withdrawal (10 µg/kg and less). No residues were found in kidney at any time.

Pigs

In a non-GLP compliant study, (Moran et al., 1992) the concentrations of narasin residues were determined in the muscle and liver tissues of pigs (12 barrows and 12 female) fed with a finishing ration containing 0 or 45 mg narasin/kg *ad libitum* for 14 days. The animals were assigned to four pens with equal numbers of each sex. Tissues were collected at 12 and 24 hours withdrawal time and were analyzed for the presence of narasin. No residues at or above the limit of quantification of the method (LOQ = 25 µg/kg) were observed in the tissues of any animals sacrificed at either hours.

Chickens

Three GLP compliant studies were conducted to evaluate residues of unlabelled narasin in the edible tissues of chickens.

In the first study (Lacoste and Larvor, 2003), 32 Ross broilers chickens (an equal number of male and females) were fed 80 mg narasin/kg feed for 5 consecutive days. Birds were housed in communal cages on a slatted wire floor in groups of four (assigned in cages by sex). Birds were slaughtered and tissue samples were taken at 0, 6, 12, and 24 h withdrawal time. Narasin was quantified by HPLC with UV detection after post-column derivatization. The limit of quantification (LOQ) was 25 µg/kg and limit of detection (LOD) was 10 µg/kg. Narasin was not detected at zero h withdrawal time in muscle and kidney. In liver and in skin/fat, narasin was not detected at 6 hours and 24 hours withdrawal time. The results are represented in Table 13.

**Table 13: Narasin residues in chicken tissues.**

| | | Mean concentration (µg/kg) | | | |
|---|---|---|---|---|---|
| Withdrawal Time (hours) | Number of. Chickens | Muscle | Liver | Kidney | Skin/Fat |
| 0 | 8 | ND | 46.2 | BLQ | 67.1 |
| 6 | 8 | ND | ND | ND | 39.1 |
| 12 | 8 | ND | ND | ND | BLQ |
| 24. | 8 | ND | ND | ND | ND |

BLQ: Below limit of quantification
ND: Not detected

In the second study (Maruyama and Sugimoto, 2000), broiler chickens were fed a medicated ration from day 0 to day 42. Three groups of birds were used, one control group (non-medicated feed), the second group was fed with medicated feed containing 80 mg narasin/kg and the third group fed with medicated feed containing 160 mg narasin/kg. Nine chickens per group were slaughtered by exsanguination. Tissue samples were taken at day 21 during medicated feed administration and at 42 days, at 2, 24, 72, 120 and 168 hours withdrawal. Muscle, liver, kidney skin and fat samples were taken. Narasin residues were determined by bio-autography using *Bacillus stearothermophilus* var. *calidolactis* C-953 as the indicator organism (Limit of quantification = 25 µg/kg).

In the 80 mg narasin/kg dose group, narasin residues were quantified in fat and skin at 2 and 24 hours withdrawal time, respectively. In the other tissues, there were no quantifiable narasin residues in any of the withdrawal times. The results are shown in Table 14. In the 160 mg narasin/kg dose group, narasin residues were quantified in higher concentrations in fat and skin at 2 hours withdrawal. In all

tissues, narasin was not quantified at 24 hours with the exception of skin (72 hours). The results are shown in Table 14.

**Table 14: Residues in chicken tissues (mg/kg) using 80 mg/kg medicated feed.**

| Test Groups | Sampling Point | Sample No. | Muscle | Liver | Kidney | Fat | Skin |
|---|---|---|---|---|---|---|---|
| N A R A S I N 80mg/kg | Day 21 | 1 | <0.025 | <0.025 | <0.025 | 0.15 | 0.09 |
| | | 2 | <0.025 | <0.025 | <0.025 | 0.14 | 0.15 |
| | | 3 | <0.025 | <0.025 | <0.025 | 0.09 | 0.17 |
| | | Average | | | | 0.13 | 0.13 |
| | 2 hours[1] | 4 | <0.025 | <0.025 | <0.025 | 0.09 | 0.05 |
| | | 5 | <0.025 | <0.025 | <0.025 | 0.06 | 0.03 |
| | | 6 | <0.025 | <0.025 | <0.025 | 0.13 | 0.04 |
| | | Average | | | | 0.09 | 0.04 |
| | 24 hours[1] | 7 | <0.025 | <0.025 | <0.025 | <0.025 | <0.025 |
| | | 8 | <0.025 | <0.025 | <0.025 | <0.025 | 0.03 |
| | | 9 | <0.025 | <0.025 | <0.025 | <0.025 | 0.03 |
| | 72 hours[1] | 10 | | | | <0.025 | <0.025 |
| | | 11 | | | | <0.025 | <0.025 |
| | | 12 | | | | <0.025 | <0.025 |
| | 120 hours[1] | 13 | | | | | <0.025 |
| | | 14 | | | | | <0.025 |
| | | 15 | | | | | <0.025 |

[1] Samples times post treatment at 42 days

**Table 15: Residues in chicken tissues (mg/kg) using 160 mg/kg medicated feed.**

| Test Group | Sampling Point | Sample No. | Muscle | Liver | Kidney | Fat | Skin |
|---|---|---|---|---|---|---|---|
| N A R A S I N 160mg/kg | Day 21 | 21 | <0.025 | <0.025 | <0.025 | 0.21 | 0.51 |
| | | 22 | <0.025 | 0.029 | <0.025 | 0.20 | 0.47 |
| | | 23 | <0.025 | 0.026 | <0.025 | 0.20 | 0.35 |
| | | Average | | | | 0.20 | 0.44 |
| | 2 hours[1] | 24 | <0.025 | <0.025 | <0.025 | 0.19 | 0.10 |
| | | 25 | <0.025 | <0.025 | <0.025 | 0.12 | 0.07 |
| | | 26 | <0.025 | <0.025 | <0.025 | 0.17 | 0.09 |
| | | Average | | | | 0.16 | 0.08 |
| | 24 hours[1] | 27 | <0.025 | <0.025 | <0.025 | <0.025 | <0.025 |
| | | 28 | <0.025 | <0.025 | <0.025 | <0.025 | <0.025 |
| | | 29 | <0.025 | <0.025 | <0.025 | <0.025 | 0.032 |
| | 72 hours[1] | 30 | | | | <0.025 | <0.025 |
| | | 31 | | | | <0.025 | <0.025 |
| | | 32 | | | | <0.025 | <0.025 |
| | 120 hours[1] | 33 | | | | | <0.025 |
| | | 34 | | | | | <0.025 |
| | | | | | | | <0.025 |

[1] Samples times post treatment at 42 days

In the third study (Handy, et al., 1985), one day-old Hubbard X White Mountain broiler chicks were fed for at least 45 days with a ration containing 80 mg narasin/kg. Four male and four female birds were slaughtered at each sampling time. Skin with adhering fat and abdominal fat tissue samples were collected after 6, 12, 18 and 28 hours withdrawal time. The analyses were realized by bio-autographic

assay using *Bacillus subtilis* as the indicator organism. The limit of detection was 5 µg/kg. Concentrations above the limit of detection were found up to 28 hours withdrawal time. No statistical differences in residue concentration due to sex were observed.

## METHODS OF ANALYSIS

For detection of narasin residues different methods have been described.

*Screening methods*

In a GLP compliant study (Maruyama and Sugimoto, 2000), screening by thin layer chromatography - bio-autography has been developed. The extraction procedure for tissue samples is based on solvent extraction with acetonitrile and n-propanol and further purification using a Sep-Pack silica cartridge. The bio-autography was performed by melting agar over the surface of the TLC plate seeded with *Bacillus stearothermophilus* var. *calidolactis* C-953 innoculum. After incubation for 18 hours at 56 °C, the zones of inhibition were measured to determine narasin presence. The limit of quantification (LOQ) was estimated considering 2.0g of sample, 0.5ml final volume of sample solution and minimal concentration of standard solution of 0.1µg/mL.. The LOQ was 25 µg/kg: Recovery from tissues was tested by the addition of 0.4 µg of narasin standard to the 2.0 g the control tissues. At this concentration, the recoveries were 84 – 100 %. The authors reported that the calibration curves showed good linearity within the tested concentrations of 0.1 - 3.2mg/kg. The accuracy, precision and the limit of detection (LOD) of the assay were not given.

In another GLP compliant screening study (Handy, et al, 1985), a TLC-bio-autographic method, using *Bacillus subtilis* as the indicator organism, was described. For this method, the limit of detection was 5 µg/kg.

A Time-Resolved Fluorescence Immunoassay (TR-FIA) screening method for the detection of narasin was developed in a non - GLP compliant study (Peippo et al., 2004). With this method, the muscle samples were treated with acetonitrile and the clean up was accomplished with an SPE silica cartridge. The eluate was reduced to dryness under nitrogen stream and reconstituted in a buffer. The resulting solution was applied to a microtiter well containing the antibody (goat anti-sheep IgG), and an aliquot of unlabelled narasin-transferrin conjugate in a reconstitution buffer was added. The plates were washed with wash solution and finally an enhancement solution was added to each plate. The time resolved fluorescence was measured by a multi-label counter. The LOD of this method was 560 µg/kg, the LOQ was 800 µg/kg. The results of the precision intra-assay and inter-assay were 3.5 and 3.6% (CV) respectively. The recovery for narasin was 89.6% with a CV of 4.1%.

*Confirmatory methods*

There are different published of HPLC and mass spectrometric methods to determinate narasin in the edible tissues of chickens:

*HPLC methods with UV vis detection*:

For these analyses, the extractions of the samples are performed with solvent and the purification is performed with a silica SPE cartridge. The sample is dried by a nitrogen stream, dissolved with a diluent solvent and transferred into a HPLC vial for analysis. The chromatographic analysis uses postcolumn derivatization with vanillin reagent, which produces a colored product that absorbs at 520 nm. (Ward et al., 2005; Lacoste and Larvor, 2003) In Table 16, the performance data are summarized.

**Table 16: Performance data for the HPLC methods with UV vis detection.**

| Criteria | Lacoste and Larvor, 2003 | Ward, et. al, 2005 |
|---|---|---|
| QA System | GLP | In house |
| Matrices | Skin/fat, muscle, liver, kidney | Skin/fat, muscle, liver, kidney |
| LOQ | 25 µg/kg | 7 µg/kg |
| LOD | 10 µg/kg | 3 µg/kg |
| Linearity | ----- | 0.9995 - 0.9999 |
| Calibration curve range | 5 - 50 µg/mL | 0.125 - 1.0 µg/mL |
| Recovery % | 77.5 – 80.6 | 76.0 - 92.6 |
| Repeatability (C.V %) | 4.1 - 6.5 | - |
| Reproducibility | | |
| Ruggedness testing | | |
| Confirmatory method | None | None |

*Mass spectrometric methods*:

Different authors have described the use of LC coupled to mass spectrometry to determine narasin in edible broiler tissues. The method included a short sample extraction and a minimal sample purification procedure. The tissues are treated with anhydrous sodium sulphate and extracted with acetonitrile and the clean up is performed with a silica SPE cartridge. The eluate is taken to dryness using nitrogen flow and then is redissolved in acetonitrile and ammonium acetate and transferred into a vial for HPLC/MS/MS analysis. The analyses are performed in the positive ion electrospray modes. The parent ion is 787, and the transitions used for the narasin confirmation are 787>431 and the 787>531. In table 17, the performance criteria of the mass spectrometric methods are shown.

**Table 17: Performance criteria mass spectrometric methods.**

| Criteria | Rokka and Peltonen, 2006 | Matabudul, et al., 2002 | Dubois, et al., 2004 |
|---|---|---|---|
| QA system | In house | In house | In house |
| Matrices | Muscle | Liver and eggs | Muscle and eggs |
| LOQ: | | 1 µg/kg | |
| LOD: | 1 µg/kg | | |
| CCα* | 1.6 µg/kg | | 0.3 µg/kg |
| CCβ* | 1.9 µg/kg | | 0.4 µg/kg |
| Linearity ($r^2$) | > 0.990 | 0.99 | |
| Calibration curve range | 1 - 5 µg/kg | 1 - 50 µg/kg | |
| Recovery % | 63 – 70 | 93 - 118 | 53 |
| Repeatability (CV %) | 5.3 - 7.0 | | |
| Reproducibility (CV %) | 12 – 27 | 6.3 - 13.7 | |
| Ruggedness testing | Not reported | Not reported | Not reported |
| Confirmatory method | Yes | Yes | Yes |

CCα: Decision Limit, CCβ: Decision Capability

The mass spectrometric methods are suitable and provide better specificity (without interference signals around the retention time) and sensitivity than do the HPLC-UV methods. Furthermore, because the methods require only a simple extraction with a short run time (about 12 min), large samples batches (more than 20 samples) can be processed daily.

## APPRAISAL

Narasin has not been previously reviewed by the Committee. It is a polyether monocarboxylic acid ionophore. It is composed of the analogues A, B, D and I. Narasin A is the major component (equivalent to 96%) and it has at least 85% of the activity. It has been classified as an anticoccidial drug in veterinary medicine and is intended to prevent and control coccidiosis caused by *Eimeria* in broilers chickens. Narasin is used at a dose range of 54 – 72 mg narasin/kg in complete feed.

Pharmacokinetics studies in both target and laboratory animals show that orally administered narasin is rapidly metabolised and eliminated within a few days. Eighty-five percent of the dose is detected in the excreta within 48 hours. Radioactivity collected from the excreta of rats and chickens shows that a low percentage (3-5%) of the recovered radioactivity is in urine and over 90% in the faeces.

Metabolism was studied in animals using $^{14}$C-radiolabelled narasin. In those studies, multiple metabolites of narasin A and narasin B have been identified in excreta. Unchanged narasin represented less than 3% of the total radioactivity. Liver metabolites are the same as those found in excreta. Hydroxylation appears to be the major route for the metabolism of narasin to polar inactive metabolites. Comparative studies indicate that the metabolite pattern is qualitatively similar among species; however there are quantitative differences. Antimicrobial activity studies against *Bacillus subtilis* indicate that hydroxylated metabolites have at least twenty times less activity than narasin A.

The radiolabelled and unlabelled depletion studies in chickens using different doses of narasin in feed and different dosing periods show that this drug is quickly metabolized and narasin disappears very rapidly from tissue. The major concentrations up to 6 hour withdrawal periods are detected in liver. At 2 hours withdrawal, residues are not detected in muscle and kidney; residues can be detected in skin/fat up to 24 hours withdrawal.

The liver is suitable as the target tissue, but for residue control purposes skin/fat also may be considered. Parent narasin is the appropriate marker residue because it is present in nearly all the edible tissues. Narasin metabolites have little or no microbiological activity *in vitro.*

Suitable analytical methods have been described for the determination and confirmation of narasin in edible tissues of chickens and pigs. These methods include HPLC with UV detection (LOQ of 25µg/kg wet tissues) that could be using for monitoring residues of narasin A in different tissues. Confirmatory methods such as HPLC/MS/MS provide good specificity and sensitivity. The monitoring of two parent-daughter transitions are enough to confirm the presence of presumptive positives for narasin residues. The calibration curve ranges of these methods present good linearity ($r^2 \geq 0.99$) and each point differs no more than 100 ± 10% of the mean of the response/concentration. For the HPLC/MS/MS method, an LOQ of 1 µg/kg wet tissues and a CCα of 0.3 and 1.6 µg/kg wet tissues have been described.

Residues in cattle may be determined using a TLC-bioautographic method. This method, while having a reported test sensitivity of 5 µg/kg, however, reports residue values only as a range (*e.g.*, 10-20; 5-10). As a result, in recommending permanent MRLs for pigs and chickens and temporary MRLs for cattle the Committee used the LOQ values for the HPLC-UV method.

## MAXIMUM RESIDUE LIMITS

In recommending MRLs for narasin in chickens and pigs and temporary MRLs for cattle, the Committee considered the following factors:

- An ADI of 0-5 µg/kg bw was established by the Committee based on a toxicological endpoint. This ADI is equivalent to up to 300 µg for a 60 kg person.
- Narasin A is a suitable marker residue in tissue.
- Metabolites exhibit little or no microbiological activity *in vitro*. Unchanged narasin represents approximately 5% of the total residues in liver.

- Liver contains the highest concentrations of residues. In fat, narasin residues persist for up to 72 h. Liver or fat (skin/fat in natural proportion, where applicable) are considered suitable choices for the target tissue.
- Residue data in the studies submitted were determined using several methods. These methods include a validated HPLC with post-column derivatization and UV detection and a validated HPLC/MS/MS. Both of these newer methods are suitable for routine monitoring.
- The analytical methods have been validated for chicken and pig tissues. The methods have not been adequately validated for cattle tissues.
- Because residue concentrations in chickens and pigs were low or non-detectable beyond 24 hour withdrawal, the MRLs recommended for fat (skin/fat where applicable) and liver are twice the LOQ of 25 µg/kg for the HPLC-UV method and the MRLs recommended for muscle and kidney are twice the LOQ of 7 µg/kg for the HPLC-UV method. Based on the limited residue data available for cattle, residues are similarly low in cattle and the recommended MRLs can be extended to cattle tissues.

The Committee recommended MRLs of 50 µg/kg for liver and fat and 15 µg/kg for muscle and kidney for chickens and pigs as narasin A. The Committee recommended the same MRLs, as temporary MRLs, for cattle.

The Estimated Daily Intake was not estimated because there were insufficient data points to calculate the median values for residues. Using the model diet and a marker:total ratio of 5%, the MRLs recommended above would result in an intake of 255 µg per person per day, which represents approximately 85% of the upper bound of the ADI.

Before re-evaluation of narasin with the aim of recommending permanent MRLs in tissues of cattle, the Committee would require a detailed description of a regulatory method, including its performance characteristics and validation data. This information is required by the end of 2010.

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