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SYNTHESIS OF ^{3}H AND ^{14}C-SCH 27899 BY FERMENTATION AND EVALUATION OF *IN VIVO* LABEL STABILITY

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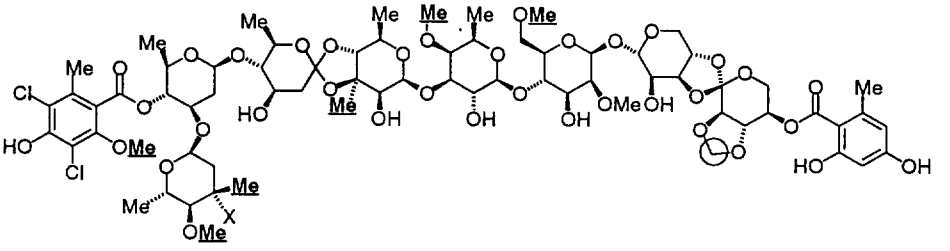
ABSTRACT

^{3}H and ^{14}C-Sch 27899 have been prepared by fermentation using the *Micromonospora carbonacea* organism. In the case of ^{3}H-Sch 27899, the label was incorporated by a single addition of 100 mCi of 70Ci/mmole L-[^{3}H-methyl]-methionine to two flasks. For ^{14}C-Sch 27899, 48 mCi of 55 mCi/mmole L-[^{14}C-methyl]-methionine was added in five aliquots to five flasks over a five day period. Both batches were isolated by solvent extraction, oxidized and purified by column chromatography and hplc. An overall incorporation of 7.8% was found from L-[^{3}H-methyl]-methionine and 18.7% from L-[^{14}C-methyl]-methionine. The *in vivo* stability of label of ^{3}H and ^{14}C-Sch 27899 was determined, with ^{14}C-Sch 27899 found to be a better choice for use in *in vivo* metabolism studies.

Key Words: Sch 27899, tritium, carbon-14, fermentation

INTRODUCTION

Sch 27899 (**1**) [1] a novel oligosaccaride from the everninomicin class of antibiotics is in Phase III clinical trials for treatment of gram positive infections which are becoming increasingly resistant to conventional antibiotic therapy.



Sch 27899 (**1**)

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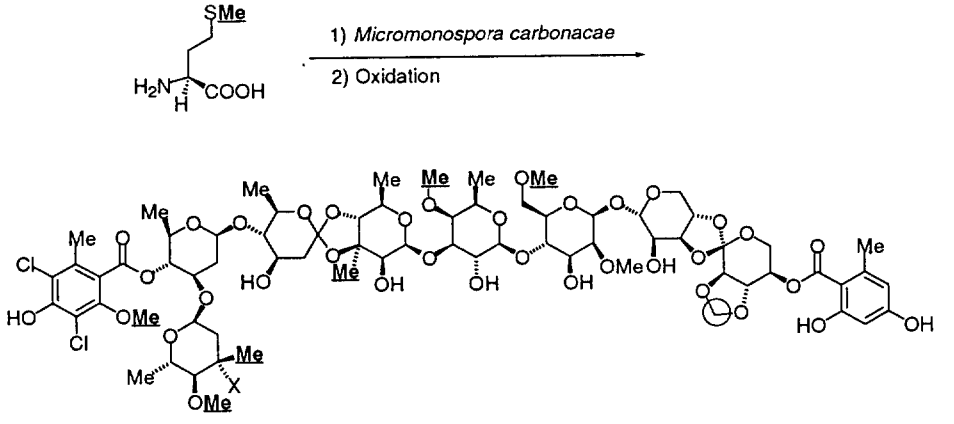
At present Vancomycin is the major therapy for infections caused by methicillin-resistant staphylococci, and is also used to treat gram positive infections in patients who cannot tolerate β-lactam antibiotics. However an increase in resistance to Vancomycin among the enterococci organism,[2] and the laboratory demonstration that this Vancomycin resistance can be transferred from an enterococcus to a stapylococcus aureus,[3] has made the discovery and development of new antibiotics, with novel mechanisms of action, increasingly urgent. Sch 27899 is not only active against Streptococcus spp.[4] and Streptococcus pneumoniae, but is also highly active against all gram positive strains resistant to Vancomycin.[5] Hence labelled Sch 27899, prepared by radiolabelled fermentation from *Micromonospora carbonacæ*,[6] was required to support the development programme.

RESULTS AND DISCUSSION

The preparation of ^{3}H or ^{14}C-Sch 27899 from L-[^{3}H or ^{14}C-methyl]-methionine is illustrated in Scheme 1. A mixture of radiolabelled Sch 27899 **1** and the nitroso analogue Sch 46459 **2** is produced by fermentation in step 1. Compound **2** is oxidized to Sch 27899 in the next step.



X = NO_2 = Sch 27899 (**1**)
X= NO = Sch 46459 (**2**)

Scheme 1

As can be seen from the structure, with one exception, the labelled methyl group is essentially incorporated intact into Sch 27899 thus in principle making ^{14}C or ^{3}H an equally good choice of label. The positions of labelling were previously determined by ^{13}C-labelling experiments and subsequently confirmed by ^{3}H nmr (Figure 2).

A major concern arising from the production of labelled Sch 27899 by fermentation is a lack of control of the site of labelling. The fact that a methylene dioxy group and O-methyl groups were labelled gave rise to stability of label concerns, and we were also uncertain on how feasible the production of sufficient quantities of ^{14}C-Sch 27899 to support compound registration would be.

Figure 2a: 320 MHz (Proton decoupled) ^3H NMR of ^3H-Sch 27899 in CD_3CN

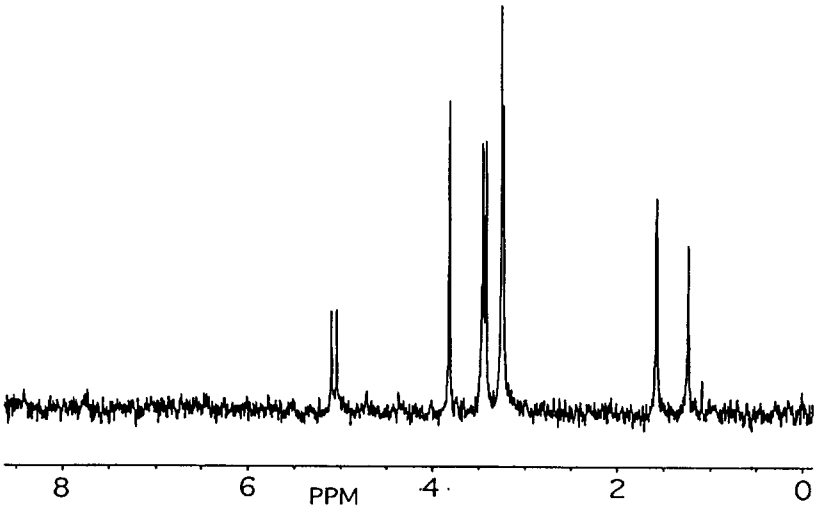
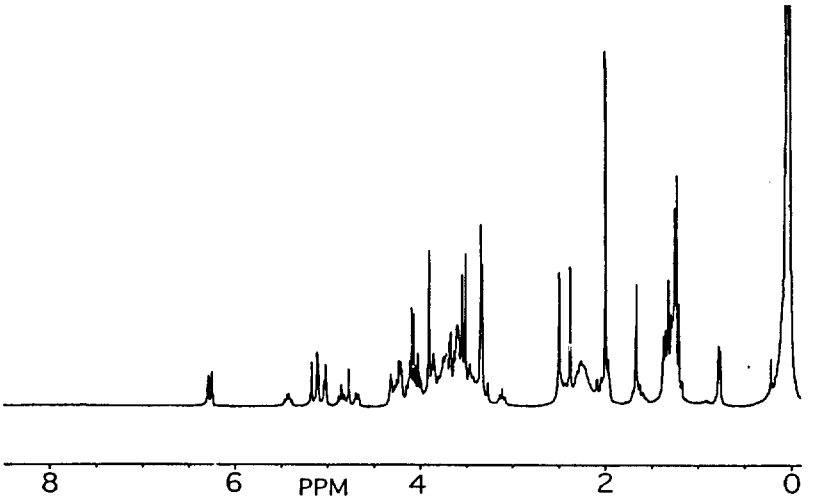


Figure 2b: 300 MHz ^1H NMR of ^3H-Sch 27899 in CD_3CN

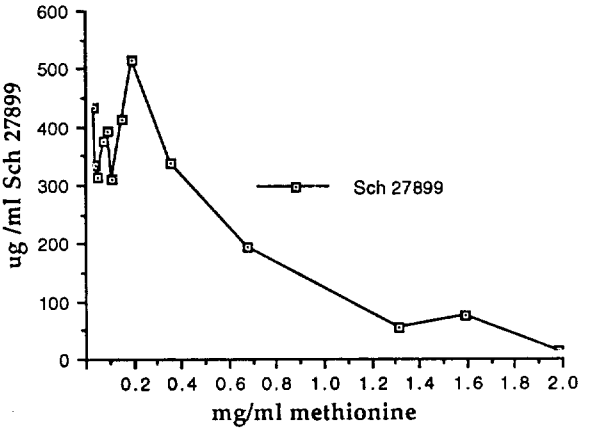


Addition of label

We had noted during initial experiments that addition of large amounts of methionine inhibited antibiotic production. A study on the effects of added methionine on antibiotic production was carried out (Figure 3).

The results showed that while antibiotic production was inhibited at relatively large concentrations of methionine (>0.7 mg/ml), the inhibition was minimal at lower concentrations, making it possible to prepare useable quantities of ^{14}C-Sch 27899 by multiple additions of ^{14}C-methionine over the course of the 5 day fermentation.

Figure 3:- Effect of L-methionine on production of Sch 27899



In contrast the much higher specific activity of ^{3}H-methionine and correspondingly smaller mass (0.002 mg/mCi for ^{3}H vs. 2.7 mg/mCi for ^{14}C) would permit addition of ^{3}H-methionine at a single timepoint.

Thus it was decided to prepare both labelled forms of Sch 27899 and conduct a formal *in vivo* stability of label study. Should the study show no difference in label stability it was planned to use the ^{3}H-Sch 27899 for the development program due to the ease of scale up with ^{3}H-Sch 27899. This would make it possible to provide enough labelled material to support the development program with a total of 2 or 3 fermentations.

Both the ^{3}H and ^{14}C-methionine precursors were added to the fermentation media as sterile aqueous solutions. The ^{3}H run used 100 mCi of 70 Ci/mmole ^{3}H-methionine, which at this specific activity could be added at one timepoint to two 100 ml fermentations. Even with the single addition, the methionine concentration was only 1 μg/ml in each flask and thus nearly three orders of magnitude below the inhibition concentration. In contrast, the 48 mCi of 55 mCi/mmole ^{14}C-methionine was added in 5 x 1.92 mCi (5.2 mg) portions to five flasks over the course of five days. Adding in this manner produced a methionine concentration of 0.05 mg/ml per addition and a total concentration of 0.26 mg/ml, which was well below the inhibition concentration. After the fermentation runs were complete, each flask was sampled for Sch 27899 titre and radiochemical purity, as shown in Tables 1 and 2.

On examination of the titres and radiochemical purity data, it is apparent that antibiotic production in the ^{14}C run was considerably better than in the tritium run. However if the control values are examined in each case, it can be seen that the difference between the two runs is independent of the label and is merely an example of the inherent variability of fermentations. Thus it is possible to speculate that a much improved yield of ^{3}H-Sch 27899 could have been achieved (15-20 mCi) had similar titre values to those found in the ^{14}C run been achieved.

Once the titres and radiochemical purities were established, the labelled Sch 27899 was extracted from the fermentation media by extraction with 9: 1 ethyl acetate: methanol, which small scale experiments had shown to give better recoveries than ethyl acetate alone.

Table 1: ^{3}H-Sch 27899

Sample	Titre in µg /ml NO + NO_2	Sp. Act. in µCi/mg NO + NO_2	% ^{3}H incorp. NO	% ^{3}H incorp NO_2
Hot 1	275	149	6.8	12.3
Hot 2	255	220	7.1	12.1
Control 1	296	-	-	-
Control 2	278	-	-	-

Table 2: ^{14}C-Sch 27899

Sample	Titre in µg /ml NO + NO_2	Sp. Act. in µCi/mg NO + NO_2	% ^{14}C incorp. NO	% ^{14}C incorp NO_2
Hot 1	869	29.9	11.5	21.0
Hot 2	1011	28.0	13.2	19.0
Hot 3	966	27.6	13.9	19.0
Hot 4	859	28.9	14.1	16.3
Hot 5	1009	24.9	14.4	19.3
Control	735	-	-	-

Oxidation Procedures

In all cases the fermentations produced significant quantities of ^{3}H-Sch 46459, the nitroso precursor of Sch 27899 (35-46%), which in the case of ^{3}H-Sch 27899 was converted to the desired nitro form of Sch 27899 by free radical oxidation using t-butyl hydroperoxide catalysed by vanadyl acetylacetonate.[7,8] On completion of the oxidation, the crude ^{3}H-Sch 27899 was cleaned up by silica gel chromatography and purified by hplc on a PVA silica column. For ^{14}C-Sch 27899, cobalt (II) acetylacetonate was used to catalyse the oxidation and a 200Å diol stationary phase was used in place of silica gel in the column chromatography step. HPLC purification was also conducted on a 200Å diol column. These process improvements when incorporated into the oxidation and purification all contributed to about a 40% improvement of the yield of ^{14}C-Sch 27899 relative to the methodology used for ^{3}H-Sch 27899. In particular the switch to cobalt acetylacetonate from vanadyl acetylacetonate improved the yield to near quantitative from 71%. Substitution of a diol stationary phase for silica gel also improved the recovery of the column chromatography step to 58% from 52%, and the use of a diol hplc column with isopropyl acetate instead of PVA silica with methylene chloride: hexane: methanol led to over 90% recovery of product instead of the 70-80% previously achieved. It is likely that the inherant acidity of PVA silica causes a reduction of yield due to the formation of the central ortho ester hydrolysis by-product.

In Vivo Stability of Label of ^{3}H and ^{14}C-Sch 27899

Radiolabelled Sch 27899 was formulated as solution with meglumine, hydroxypropyl-β-cyclodextrin (molar ratio 1:3:5), Tween 80 and mannitol. This was used to evalate the *in-vivo* stability of label in male Sprague Dawley rats after administration of a single intravenous dose of the radiolabelled formulation. The mean recovery of radioactivity is shown in Table 3:

Table 3

	Mean Percent of Administered Dose (%CV)					
Group	Urine	Faeces	Expired Volatile ^{14}C	Expired Volatile ^{3}H	Carcass	Total
^{3}H-Sch 27899	27.2 (11)	53.9 (11)	NA	0.009 (124)	7.20 (41)	88.3 (1)
^{14}C-Sch 27899	30.4 (12)	64.7 (8)	0.553 (15)	NA	5.95 (47)	101.7 (3)

It can be seen that the majority of the radioactivity was recovered in the faeces, which is consistent with high biliary excretion. Less than 1% of the radioactive dose for either isotope was recovered as volatile radioactivity. The recovery of radioactivity derived from ^{14}C-Sch 27899 was theoretical, while recovery of ^{3}H-Sch 27899 derived radioactivity was incomplete. Results from lyophilization and dessication of urine collected after an intravenous dose suggested that ^{3}H-Sch 27899 may undergo tritium exchange with total body water *in vivo*. The other difference between the two isotopes was the radioactivity recovered in the faeces, which also may be suggestive of metabolism in the GI tract to produce tritiated water or other forms of volatile tritium. In any event the results showed that ^{14}C-Sch 27899 was more suitable than ^{3}H-Sch 27899 for use in *in vivo* metabolism studies and as a result synthesis efforts were directed entirely to the production of ^{14}C-Sch 27899.

EXPERIMENTAL

Materials

L-Methyl-^{3}H-Methionine was purchased from NEN Life Science Products and L-methyl-^{14}C-methionine was purchased from Amersham plc and were used without further purification. All reagents and solvents were of commercial grade and were used as supplied.

Thin Layer Chromatography.

Thin layer chromatography was performed using Whatman LK6DF (silica gel 60) 5 x 20 cm, 0.25 mm plates. The plates were scanned on a Bioscan 1000 linear analyser. The following system was used:-

1) Methylene chloride: methanol 9: 1.

High Performance Liquid Chromatography

^{3}H and ^{14}C-Sch 27899 were analysed for chemical and radiochemical purity at various stages of the synthesis using the following hplc system:-

1) YMC ODS-A 10 cm x 4.6 mm ID, 270 nm. Methanol: 0.1 M tetramethylammonium hydroxide and 0.05 M ammonium acetate pH 7.2: water.
   Mobile Phase A: (63: 3 : 34)
   Mobile Phase B: (90: 3: 7)

Gradient Program at 1 ml/min.:-

Time (min)	% Mobile Phase A	% Mobile Phase B
Initial	93	7
5	56	44
15	56	44
24	0	100

^{3}H-Sch 27899 was purified by the following hplc system:

2) YMC 5µ 120Å PVA silica 25 cm x 1 cm ID, 254 nm. Methylene chloride: hexane: methanol (57: 40: 3), 8 ml/min.

^{14}C-Sch 27899 was purified by the following hplc system:

3) YMC 5µ 200Å Diol 25 cm x 1 cm ID, 254 nm. Isopropyl acetate, 5 ml/min.

Synthesis of ^{3}H and ^{14}C-Sch 27899

Preparation of labelled Sch 27899 by fermentation

The initial stage inoculum for this fermentation was prepared by transferring 1.5 ml of frozen *Micromonospora carbonacæ* production strain to 84 ml of the germination medium in 250 mL flasks at 30°C. The flasks were shaken at 300 rpm with a 2" stroke for 48 hours. The medium (g/L) consisted of beef extract, 3.0; tryptone, 5.0; cerelose, 1.0; potato dextrin, 24; and yeast extract, 5.0. The medium was adjusted to pH 7.3 prior to the addition of calcium carbonate, 1.0; and autoclaved for 20 minutes at 121°C. A second germination stage was prepared by adding 1.5 ml of the 48 hour culture into 250 ml flasks prepared as above. The flasks were incubated for 30 hrs at 30°C and 300 rpm.

The fermentations were run in 100 ml of medium in 500 ml Erlenmeyer flasks containing (g/L) yeast extract, 5.0; meat peptone, 6.0; corn steep powder, 2.0; potato dextrin, 60; boiled linseed oil, 4.0; cobalt chloride, 0.002; cerelose, 22; and calcium carbonate, 4.0. All ingredients except the cerelose were autoclaved together for 20 minutes at 121° C in a total volume of 90 ml, the medium being adjusted to pH 7.0 prior to the addition of the calcium carbonate. The cerelose was prepared as a filter sterilized 10X concentrate and asceptically dispensed (10 ml) before inoculation. The fermentation was initiated by transferring 1.5 ml of the second stage germination to the fermentation flasks. L-[^{3}H-methyl] methionine (70.5 Ci/mmole, 100 mCi, 6 ml) was added through a sterile filter to two flasks, before they were shaken at 400 rpm with a 1.5" stroke at 30° C for 120 hours. In the case of the ^{14}C run, 48 mCi of 55 mCi/mmole L-[^{14}C-methyl] methionine was dissolved in sterile water (15 ml) and added in 5 x 1.92 mCi, 5.2 mg, 0.6 ml aliquots through a sterile filter to five flasks at the start of the fermentation and every 24 hours for a total of 5 additions.

Isolation, Oxidation and Purification of labelled Sch 27899

^{3}H-Sch 27899

At the completion of the fermentation run, each flask was sampled for antibiotic titre and radiochemical incorporation, before they were pooled and extracted three times with two volumes of 9:1 ethyl acetate: methanol. The combined extracts were washed with sodium bicarbonate (0.3 M, 100 ml) and evaporated to dryness. About 27 mCi of crude ^{3}H-Sch 27899 was obtained.

The crude ^{3}H-Sch 27899 was dissolved in ethyl acetate (2 ml) and to this solution was added sodium bicarbonate (30 mg), vanadyl acetylacetonate (3 mg) and t-butyl hydroperoxide (5.5 M in isooctane, 190 µl). After one hour the reaction was cooled by the addition of ice chips and quenched by aqueous sodium sulphite (2 M, 1 ml). The reaction mixture was extracted with ethyl acetate (3 x 10 ml). The aqueous phase was adjusted to pH 7 and extracted with ethyl acetate (2 x 5 ml). All the organic extracts were combined and washed with aqueous sodium bicarbonate (50 g/l, 2 ml), dried over anhydrous sodium sulphate, filtered and evaporated to dryness to yield 19.3 mCi (71%) of ^{3}H-Sch 27899.

The crude ^{3}H-Sch 27899 was cleaned up by silica gel chromatography using 7.5 g of flash grade silica gel and isopropyl acetate as eluent (300 ml) followed by 90:10 ethyl acetate: hexane (200 ml) and 95:5 ethyl acetate: hexane (200 ml). Fractions containing the product were pooled and concentrated to dryness to yield 10.1 mCi. ^{3}H-Sch 27899 was finally purified by hplc using hplc system 2 to yield 7.87 mCi (7.87% Incorporation) at a specific activity of 177 µCi/mg, 288.8 mCi/mmole. Analysis on hplc system 1 showed the radiochemical purity was in excess of 98%.

^{14}C-Sch 27899

As for ^{3}H-Sch 27899, each flask was sampled for titre and radiochemical purity before being pooled and extracted with 2 volumes of 9:1 ethyl acetate: methanol. The combined extracts were washed with sodium bicarbonate (0.3 M, 100 ml) and evaporated to dryness. The residue was suspended in water (20 ml) and extracted with ethyl acetate (3 x 10 ml). The combined ethyl acetate extracts were washed with water (2 ml) and evaporated to dryness to obtain 16.4 mCi of crude ^{14}C-Sch 27899.

The ^{14}C-Sch 27899 crude was dissolved in ethyl acetate (6 ml) and to this solution was added sodium bicarbonate (50 mg), cobalt (II) acetylacetonate (3 mg) and t-butyl hydroperoxide (5-6 M in decane, 225 µl). After six hours the reaction was quenched by the addition of 6 ml of a solution containing sodium dithionite (384 mg) and dipotassium hydrogen phosphate (1.04 g). The emulsion was stirred for 10 minutes before it was transferred to a centrifuge tube. The tube was spun at 10,000 RPM for 10 minutes, and the aqueous layer removed by pipette. The ethyl acetate layer was washed with 5% sodium chloride solution (4 ml) with the separation again aided by centrifugation. The aqueous layer was removed and the ethyl acetate solution concentrated to a yellow oil to yield 16.16 mCi (98.5%) of ^{14}C-Sch 27899.

The crude ^{14}C-Sch 27899 was initially purified by gravity chromatography using 20 g of 50µ 200Å diol stationary phase with isopropyl acetate as eluent. Fractions containing the product were pooled and concentrated to dryness to yield 9.5 mCi. ^{14}C-Sch 27899 was finally purified by hplc using hplc system 3 to yield 9.0 mCi (18.7% Incorporation) at a specific activity of 27 µCi/mg, 44 mCi/mmole. Analysis on hplc system 1 showed the radiochemical purity was in excess of 96%.

In Vivo Stability of ^{3}H-Sch 27899 and ^{14}C-Sch 27899 in Male Rats

^{3}H-Sch 27899 and ^{14}C-Sch 27899 were formulated in a 1:3:5 molar ratio with meglumine (NMG) and hydroxypropyl-β-cyclodextrin (HPβCD) in sterile water containing Tween 80 (0.57 mg/ml) and mannitol (28.5 mg/ml).

Male Sprague Dawley rats were fasted overnight and then given a single intraveneous dose of ^{3}H-Sch 27899 or ^{14}C-Sch 27899 at 15mg/kg via the jugular vein. Immediately after IV administration, rats were placed in glass metabolism cages equipped with 2 column reservoirs (tower 1 and 2) each containing appropriate trapping solutions. The trapping solutions used for ^{3}H-Sch 27899 was 150 ml Monophase® in tower 1 and 100 ml ethylene glycol in tower 2. Additionally the cage set up also included a 20 g charcoal filter and a cold trap condensor. For ^{14}C-Sch 27899, 150 ml of Carbosorb® : 2-ethoxyethanol (1:1; v:v) was used in both towers.

Urine was collected in block intervals at 0-8, 8-24 and 24-48 hr post-dose and faeces were collected in block intervals at 0-24 and 24-48 hr post dose. Trapping solutions were collected in block intervals at 0-4, 4-8, 8-24 and 24-48 hr after dose administration. The reservoirs were filled with fresh trapping solution following each block collection. Charcoal filter and cold trap condensates were collected 48 hr after dose administration.

Trapping solutions, urine (aliquots analysed before and after drying) and cage rinses were analysed for radioactivity by liquid scintillation counting. Faeces were homogenized with water and oxidized using a Packard Tricarb Model 307 Tissue Oxidizer. The oxidized residue was then analysed by liquid scintillation counting.

Carcasses were digested with 6M potassium hydroxide solution and analysed by liquid scintillation counting. Charcoal samples were oxidized and quantitated by liquid scintillation counting in a similar manner to the faecal samples.

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