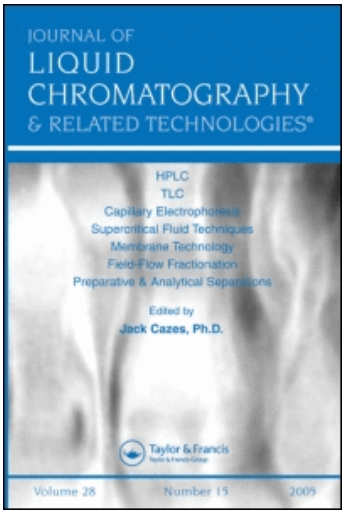

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Purified ^{14}C Virginiamycin

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PURIFIED ^{14}C VIRGINIAMYCIN: SEPARATION, PURIFICATION, AND RECOMBINATION OF ^{14}C FACTORS S AND M

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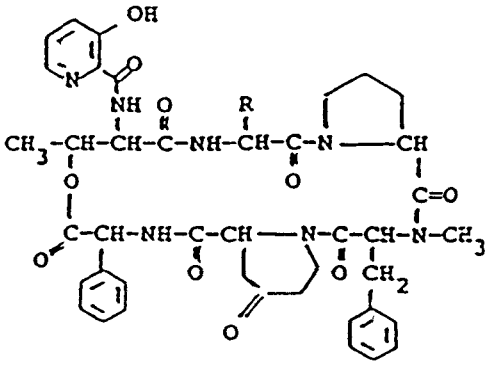
ABSTRACT

^{14}C virginiamycin, an antibiotic composed of two synergistic factors S and M, was required for metabolism and residue studies. ^{14}C Factors S and M were separated from crude ^{14}C virginiamycin and purified by semi-preparative normal phase HPLC followed by crystallization/precipitation. After chemical and radiopurity assays, the two factors were recombined in a 1:4 ratio to form purified ^{14}C virginiamycin.

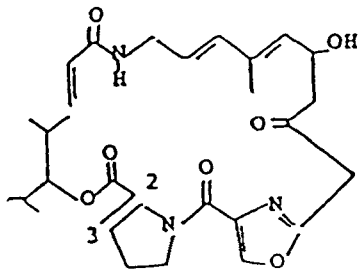
INTRODUCTION

Virginiamycin, an antibiotic produced by Streptomyces virginiae, is currently used by SmithKline Beckman Animal Health Products as a growth promotant.[1] It is a mixture of microbiologically synergistic factors S and M.[2]

Factor S



Factor M_1



S_1: R = C_2H_5

Tissue residue and metabolism studies requested by the FDA require high purity ^{14}C labelled virginiamycin. For previous studies ^{14}C virginiamycin had been prepared by multiple extractions on a Craig countercurrent extractor.[3] This purification technique resulted in only 96% radiopurity material. This paper describes the separation, purification, recombination, and assay of the radioactive factors to provide 98% pure ^{14}C virginiamycin needed for the studies.

MATERIALS AND METHODS

Preparative Apparatus

The preparative chromatography was done using three Rainin Rabbit HPX pumps with 50 mL heads, a MacRabbit Controller, a Knauer 87 UV Detector (330 nm, 0.4 mm cell path, 0.32 range), a Gilson 201C fraction Collector, and a Hewlett Packard 3390 A Integrator. A Rainin Dynamax Macro HPLC Silica column 8u, 21 mm x 25 cm with a Dynamac Silica Guard Module, 21.4 mm x 5 cm was used.

Preparative Experimental

The starting material for the preparative chromatography had a purity of 68%. A series of extractions on the whole broth had been performed at SmithKline-RIT with methyl isobutyl ketone followed by precipitation with hexane.

The virginiamycin was then dissolved in chloroform and filtered (0.2u). Up to an 800 mg sample was injected per run via the third pump. The solvent flow was 30 mL/minute and the mobile phase was chloroform (one minute) and 1.75% methanol/chloroform (19 minutes). After every six injections the column was washed with 20% methanol/chloroform to remove polar impurities. The factors S and M were separately collected (see figure 1) and stored over dry ice. The mobile phase was flash evaporated below 35°C. The Factor S containing fraction, a gummy solid, was recrystallized in methanol, dried, and assayed. Factor M was recycled through the preparative HPLC using the same conditions. After flash evaporation, the factor M was dissolved in minimal chloroform, precipitated with six volumes petroleum ether, filtered, dried, and assayed.

After the necessary purity was achieved, the two ^{14}C factors S and M were combined in a 1:4 ratio by weight and equal specific activity. This was accomplished by dissolving the proper amounts in minimal chloroform, then slowly precipitating with six volumes of petroleum ether. The resulting ^{14}C virginiamycin was filtered, dried, and assayed.

Analytical Apparatus

For the analytical chromatography needed for the chemical and radiopurity assays, the same instrumentation described previously was

used with the addition of a Rheodyne Injector Valve equipped with a 20 uL or 100 uL loop. The wavelength was changed to 297 nm, the cell path to 10 mm, and the range to 0.64-2.56. An E.S. Industries Lichrosorb Si 60 column (4.6 mm x 25 cm) was used.

A Bioscan Linear Analyzer was used for the TLC radiopurity assay and a Packard 460 Liquid Scintillation Counter was used both for the radiopurity analyses of aliquots collected from the HPLC assay and for the specific activity determination.

Analytical Experimental

For the chemical purity assay, solvent flow was 2.0 mL/min and the mobile phase was 98:2:0.02 chloroform: methanol: trifluoroacetic acid. Decreasing the column temperature to <0°C increased column efficiency. Chemical purity was determined from a three-point standard curve using the appropriate factor reference standard.

For the radiopurity HPLC assay, the same conditions were used. The eluate was collected in scintillation vials every ten seconds. The mobile phase was evaporated and the appropriate scintillation cocktail added. After correcting for background, a histogram of radioactivity versus time was used to determine the radiopurity.

For the radiopurity TLC assay, Baker silica gel HPF 200 um 10 cm x 10 cm plates were used with the two solvent systems

- chloroform: methanol 93:7
- ethyl acetate: methanol: ammonium hydroxide 10:1:0.5

For specific activity determination, triplicate samples of the compound were radioassayed using ^{14}C hexadecane as the internal standard.

Reagents

Ammonium hydroxide, Fisher
Chloroform, Burdick and Jackson, HPLC
Ethyl acetate, Baker, HPLC
Methanol, Baker, HPLC
Petroleum ether, Baker 3-9268
Trifluoroacetic acid, Baker, A.R. 99.6%
Atomlight, New England Nuclear
Dimilume® 30, Packard
^{14}C Virginiamycin; prepared by fermentation using a mixture of ^{14}C amino acid precursors at SmithKline-RIT, Rixensart, Belgium

RESULTS

After recrystallization, ^{14}C Factor S had a chemical purity of 100% as compared to reference standard, radiopurity by HPLC/TLC of 99%, and a specific activity of 6.0 mCi/g. After precipitation, ^{14}C Factor M had a chemical purity of 100% as compared to reference standard, a radiopurity by HPLC/TLC of 98%, and a specific activity of 2.0 mCi/g.

After recombination of the ^{14}C factors S and M, virginiamycin had a chemical purity of 100% as compared to the respective reference standards, a radiopurity by HPLC/TLC of 98%, and a specific activity of 2.07 mCi/g. From 34.6 g of starting material containing 23.6 g of virginiamycin, 17.2 g of purified ^{14}C virginiamycin was recovered in an overall yield of 73%.

Following are chromatograms of the starting material on the preparative column (figure 1) showing where the cuts were made and the final product on the analytical column (figure 2).

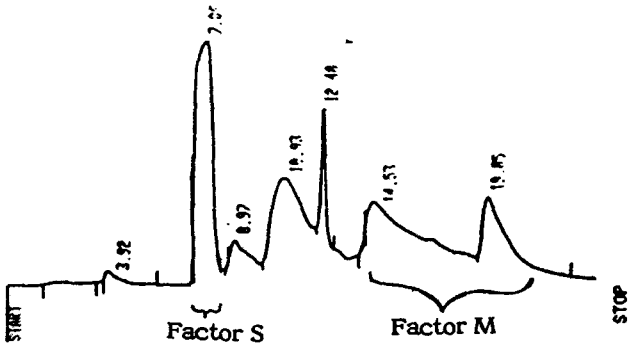


Figure 1 - Starting Material (preparative)

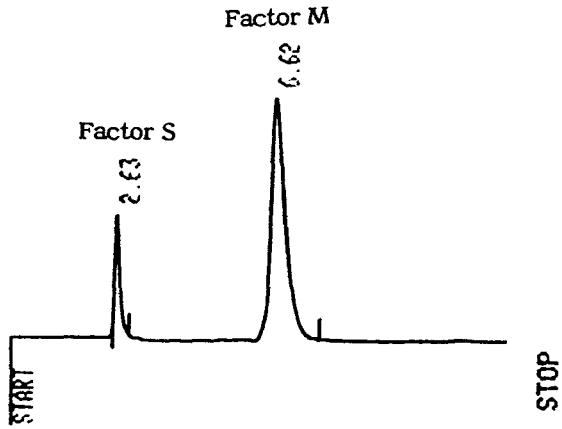


Figure 2 - Purified ^{14}C virginiamycin (analytical)

DISCUSSION

The greatest difficulty with the purification was the instability of the ^{14}C materials, especially factor M. During the chromatographic runs, all fractions while being collected were stored over dry ice. As described in the experimental section, they were flash evaporated with a bath temperature below 35°C. After drying, all samples were immediately stored at -80°C. Trifluoroacetic acid, used for the analytical chromatography because it helped improve resolution, had to be omitted during preparative chromatography because of factor M instability.

Before the purified ^{14}C factors S and M could be combined, the specific activities of the two had to be equalized. In this case since factor S had a higher specific activity, it was diluted with an appropriate amount of cold reference standard factor S. The 1:4 addition ratio of the two factors S and M falls within the optimal range of the microbiological synergy.

ACKNOWLEDGEMENTS

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