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Extraction of Premafloxacin Radiolabeled with Carbon Fourteen from Loam Soil

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EXTRACTION OF PREMAFLOXACIN RADIOLABELED WITH CARBON FOURTEEN FROM LOAM SOIL

by

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Western Michigan University, 1997

Premafloxacin is a fluoroquinolone antibiotic being developed by Pharmacia & Upjohn for veterinary indications. Since the parent drug and possibly metabolites will be applied to soil through the deposition of animal excreta, many regulatory agencies will require environmental assessment testing. In order to determine if ^{14}C-premafloxacin would biodegrade in the soil, loam soil was extracted with solvents and C18 solid phase columns were used to isolate the compound after three weeks of incubation. In addition to extracting the soil, production of $^{14}CO_2$ was monitored by trapping the degradation by-product in 2 N NaOH. Liquid scintillation counting of the solvent extracts and trapping solution appeared to indicate a high binding affinity between ^{14}C-premafloxacin and the soil. This affinity can reduce the bioavailability of the drug to microorganisms thus inhibiting aerobic biodegradation. Contact with loam soil will therefore immobilize the drug and naturally occurring environmental conditions involving weak acid, base and saline solutions will not dissociate the drug from the soil.

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INTRODUCTION

In the United States, all drugs intended for use in animals are regulated by the Food and Drug Administration's Center for Veterinary Medicine. The agency requires environmental fate and effects studies for some veterinary drugs in accordance with the FDA's Environmental Assessment Technical Assistance Handbook (FDA, 1987). If the environmental assessment (EA) proves acceptable, a Finding Of No Significant Impact (FONSI) is issued and the EA is made available to the public.

The value of this testing is currently being questioned by several countries. In the United States, the basis of the controversy is supported by reviewing past EA submissions. The EA regulations were implemented in 1985. Since 1985, no product has ever been refused marketing authorization because of a concern regarding the environmental fate and effects of the drug substance stemming from its intended use (Robinson, 1996). Resolution of this issue will undoubtedly take years so EA testing for veterinary medicinal products will continue to be required by many countries prior to worldwide marketing. Premafloxacin is a broad spectrum fluoroquinolone antibacterial agent with both gram positive and gram negative activity. It is being

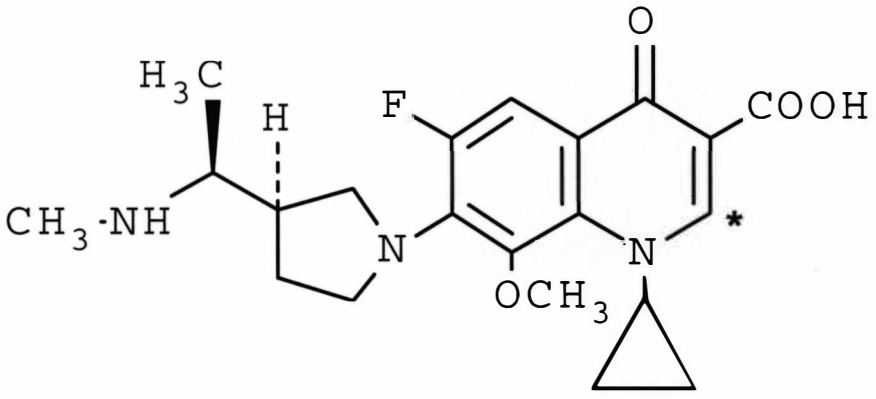
developed in the Pharmacia & Upjohn, Inc. Animal Health associated business for nonspecific infections in both companion animals and livestock. Mechanisms for the environmental degradation of plant alkaloids containing quinolines have been identified (Abbott Laboratories, 1995) therefore it is possible that premafloxacin could biodegrade because it is a substituted quinoline compound. The drug was isolated from the soil by solvent extractions and a liquid scintillation counter was used to determine the extraction efficiencies of the solvents. Soil, incubated with the drug for three weeks, was extracted after a solvent was selected. This final extract was analyzed using high performance liquid chromatography with radioactive monitoring (HPLC-RAM) to estimate ^{14}C-premafloxacin biodegradation. A 2 N NaOH solution was used to trap $^{14}CO_2$ evolving from the soil. The gas represents the final most mineralized form of a molecule and can signal the degradation of the drug. This thesis describes the development of an extraction process which demonstrates that ^{14}C-premafloxacin has a high binding affinity for the loam soil used in this study.

METHODOLOGY

Materials

Radiolabeled Premafloxacin

A carbon 14 atom was introduced into premafloxacin in the 2-carbon position of the quinolone ring by Amersham Life Science, Inc., Arlington Heights, IL. The chemical name is [S-(R^*,S^*)]-1-cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-7-[3-[1-(methylamino)ethyl]-1-pyrrolidinyl]-4-oxo-3-[2-^{14}C]-quinolinecarboxylic acid. The molecular formula is $C_{21}H_{26}FN_3O_4$ and the molecular weight is 405.1 for the specified specific activity. The amphoteric compound has pK_a's at 6.5 and 10. The lot number was CFQ8396 and the drug had a radiochemical purity of 91% to 95% according to a previous HPLC-RAM purity evaluation. The specific activity of the drug was 128.4 µCi/mg and the structure is illustrated in Figure 1 on the following page.



* Location of 14C radiolabel

Figure 1. Structure of ^{14}C-Premafloxacin.

Chemicals

The following chemicals were used:

1. EM Science, Gibbstown, NJ.- acetonitrile (CH_3CN), methanol (MeOH), HPLC grade water.

2. J.T. Baker, Phillipsburg, NJ.- potassium hydroxide 45% (w/w) solution (KOH), polyethylene glycol 300, 2.0 N sodium hydroxide (NaOH).

3. Aldrich, Milwaukee, WI.- diethylamine (DEA), triethylamine (TEA), trifluoroacetic acid (TFA), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), 4-methyl-2-pentanone, n-

methyl ethyl ether.

4. Pierce, Rockford, IL.- n-heptafluorobutyric acid (anhydrous)(HFBA).

5. Mallinckrodt, Paris, KY.- sodium chloride (NaCl), sodium citrate dihydrate, citric acid monohydrate, acetic acid, calcium chloride dihydrate ($CaCl_2$), copper sulfate ($CuSO_4$), ammonium hydroxide (NH_4OH), hydrochloric acid (HCl).

6. Sigma, St. Louis, MO.- D-(+) glucose anhydrous, pipemidic acid.

7. Eastman Kodak, Rochester, NY.- sodium dodecyl sulfate (SDS), 1-hexane sulfonic acid sodium salt, tetrabutylammonium hydroxide titrant (0.4 M in water) HPLC grade (TBA).

8. Baxter Health Corporation Burdick & Jackson Division, Muskegon, MI.- chloroform, methylene chloride, n-butyl chloride.

9. Aaper Alcohol and Chemical, Shelbyville, KY.- ethyl alcohol USP absolute (EtOH).

10. Alconox, New York, NY.- Liqui-Nox.

11. Isolab, Akron, OH.- Isoclean.

Loam Soil

Loam soil is soil consisting mainly of sand, clay, silt, and organic matter. This type of soil was obtained from a Richland, MI field and was used for each experiment involving soil.

HPLC-RAM Instrumentation

A HPLC system (Perkin Elmer Corp., Norwalk, CT.) was used for analysis of the standard and final extract. This included a Series 200 quaternary gradient pump, ISS-200 autosampler, LC-235C diode-array UV detector and TurboChrom 4 instrument control software. The HPLC column was a Phenomenex Prodigy-5 C18, 3 mm x 150 mm equipped with a similar guard column. Mobile phase A was a 100 mM citrate buffer at pH 4.4 and mobile phase B was UV grade acetonitrile. The pump conditions began with an equilibration at 85% A and 15% B. These conditions were maintained for ten minutes to elute polar metabolites. The next step was a gradient which changed A to 60% and B to 40% after 10 minutes. The drug was eluted during this time. In order to elute less polar metabolites, 60% A and 40% B was continued for ten additional minutes. The HPLC flow was 0.5 mL/min and the UV detector was set to 300 nm with a 50 µL injection volume.

The radioactive monitor (RAM) was a Model A200 from Radiomatic Instruments/Packard (Packard Instrument Co., Meriden, CT.) equipped with a 0.5 mL flow cell with windows set at 0-160 KeV. The scintillation cocktail in the RAM was Ultima-Flo M from Packard pumped at a rate of 1.5 mL/min.

Liquid Scintillation Analyzer

All extracts were analyzed using a Packard 1900TR Tri-Carb Liquid Scintillation Analyzer. This instrument was serviced before the study and checked periodically with a ^{14}C-standard. Packard Insta-Gel XF and Hionic-Fluor liquid scintillation cocktails were used for this study. The scintillation data was expressed as disintegrations per minute (DPM).

Solid Phase Extraction Columns

The solid phase extraction columns were supplied by Varian Sample Preparation Products (Harbor City, CA.) in 50 mg to 1 g sizes. The sample reservoir was from Supelco (Bellefonte, PA.).

RESULTS AND DISCUSSION

Experimental Procedures

^{14}C-Premafloxacin Glucose Stock Preparation

A 5.00 mg quantity of ^{14}C-premafloxacin was accurately weighed on a balance and transferred into a 1000 mL volumetric flask. HPLC grade distilled water was used to dissolve the solute making the concentration 5 µg/mL. Glucose (1.0 g) was dissolved using 200 mL of this 5 µg/mL solution which produced the ^{14}C-premafloxacin glucose stock used for the various experiments throughout this study. The glucose is used to provide a carbon source for the soil microorganisms. Scintillation analysis of the stock showed that the actual concentration was 3.75 µg/mL when compared to the theoretical value obtained using the weight and specific activity for the drug. This reduction was due to the initial purity of 91% to 95% and from loss of drug due to glass binding which has been observed in previous toxicokinetic assay development work.

Stock Analysis

The 5 µg/mL ^{14}C-premafloxacin glucose stock was analyzed using a

scintillation counter and HPLC-RAM on the day it was prepared. These values were compared with the DPM values from the three week incubated soil extraction on page 34 to determine an extraction efficiency. Scintillation analysis of 50 µL of this stock produced 54,423 DPM and HPLC-RAM analysis produced 15,586 DPM.

Extraction Method Development

Two grams of soil were extracted for most experimental conditions. This amount was used to reduce radioactive waste production. The soil was weighed into 50 mL polypropylene screw cap tubes then 100 µL to 200 µL of the 5 µg/mL ^{14}C-premafloxacin glucose stock were added. The soil was extracted immediately after addition of the stock by dispensing a 10 mL aliquot of solvent into the tubes then the tubes were placed on a mechanical rocker for 20 minutes. The tubes were centrifuged using a Beckman J2-MC at 2,000 RCF for 8 minutes. A 1 mL portion of the supernatant was added to 18 mL of scintillation cocktail, mixed, and analyzed using liquid scintillation counting. The extract was compared to the same amount of stock before extraction to determine the percent recovery of the extract. This procedure provided a rapid way of determining if the drug was being extracted into the solvent.

Solvent Selection Process

Acidic Ion Pairing Agents

An attempt to extract ^{14}C-premafloxacin into an organic solvent directly from the soil was made by using acidic ion pairing compounds. Trifluoroacetic acid (TFA), heptafluorobutyric acid (HFBA), and acetic acid can form ion pairs with ^{14}C-premafloxacin and reduce the interactions between the soil matrix and the drug thus promoting the extraction into an organic solvent. Different percentages of the ion pairing agents were added to 10 mL portions of 80/20 CH_3CN:EtOH. Stock solution (200 µL) was added to 2 gram portions of soil in 50 mL polypropylene tubes. Each tube was extracted with 10 mL of the extraction solvent then analyzed using a scintillation counter. The stock solution (200 µL) was also added to 1 mL of 1 M HCl in sat. NaCl. This was the standard used for comparison with the extracts. A halogen was added to the standard so the extracts and standard both contained halogens. This was done to help compensate for possible quenching effects. After this experiment, the standard was blank soil extracted with 1 M KOH spiked with the stock solution. This provided a background similar to many of the extracts. The 10% TFA solution had the highest efficiency with approximately 19% of the ^{14}C-premafloxacin recovered. The TFA increased the extraction efficiency over the initial

extraction but the recovery was still too low. The results are shown in Table 1.

Table 1

Scintillation Data From the Acidic Ion Pairing Extractions

Solvents	DPM	Recovery (%)
200 µL stock + 1 mL 1 M HCl in sat. NaCl	188771	-----
10% TFA in 8/2 CH_3CN:EtOH	35180	19
1% TFA in 8/2 CH_3CN:EtOH	10910	6
10% HFBA in 8/2 CH_3CN:EtOH	29860	16
1% HFBA in 8/2 CH_3CN:EtOH	11170	6
10% acetic acid in 8/2 CH_3CN:EtOH	4480	2

Stock = 5 µg/mL ^{14}C-premafloxacin/glucose TFA = trifluoroacetic acid HFBA = n-heptafluorobutyric acid

Aqueous Extractions

The low solubility of ^{14}C-premafloxacin in organic solvents prompted the investigation of aqueous extractions with a concentration step using solid phase extraction. Salt solutions were used to reduce ionic interactions. Organic compounds like EDTA were added to chelate metals and bind to lignin, humic substances, and other organic soil constituents. Blank soil was extracted with 1 M KOH which provided a dark blank background that was spiked with 100 µL of the stock solution

and used for comparison with the extracts. A solution of 75% 100 mM citrate buffer pH 4.4 with 25% CH_3CN yielded an efficiency of 15%. EDTA solutions had recoveries of less than 5%. Low recoveries from the acidic extractions indicated that protonation of the carboxylic acid group of the drug did not decrease interactions with the soil matrix. Some results are listed in Table 2.

Table 2

Scintillation Data From the Aqueous Extractions

Solvents	DPM	Recovery (%)
100 µL stock + 1 mL blank soil extract (extracted with 1 M KOH)	59759	-----
75/25 100 mM citrate:CH_3CN	8750	15
1 M HCl in sat. NaCl	5280	9
2 M $CaCl_2$ in 10 mL H_2O	3260	6
$CuSO_4$ (saturated)	2100	4
1 mg pipemidic acid in 10 mL H_2O	2060	3

Stock = 5 µg/mL ^{14}C-premafloxacin/glucose

Basic Aqueous Extractions

Basic solutions above pH 10 ionize the carboxylic acid group and deprotonate the amine. This form of the drug showed much better extractability into aqueous solutions. A 0.1 M KOH solution yielded 43% recovery and a 0.4 M tetrabutylammonium hydroxide (TBA) solution

showed 34% recovery. Although the percent recoveries were higher, the extracts were very dark because the basic solutions dissolved more of the soil matrix. This made the solid phase extraction procedure more difficult. Additional solvents and recoveries are listed in Table 3.

Table 3

Scintillation Data From the Basic Aqueous Extractions

Solvents	DPM	Recovery (%)
100 µL stock + 1 mL blank soil extract (extracted with 1 M KOH)	59759	-----
0.1 M KOH	25640	43
1 M KOH	24170	40
0.4 M TBA	20470	34
10% TEA	18740	31
10% DEA	15860	27
1 M NH_4OH	11930	20

Stock = 5 µg/mL ^{14}C-premafloxacin/glucose
TBA = tetrabutylammonium hydroxide titrant
TEA = triethylamine
DEA = diethylamine

Basic Detergent Extractions

Detergent compounds which can form ion pairs with the drug were tested in 0.1 M KOH containing ethanol or methanol. Most of these compounds were selected because they have a strong interaction with soil. A solution containing hexane sulfonic acid produced a recovery of

61% and some non-detergent solutions were also used which are presented in Table 4.

Table 4

Scintillation Data From the Basic Detergent Extractions

Solvents	DPM	Recovery (%)
100 µL stock + 1 mL blank soil extract (extracted with 1 M KOH)	59759	-----
30% EtOH + 10 mg/mL hexane sulfonic acid in 0.1 M KOH	36200	61
30% EtOH sat. with sodium dodecyl sulfate + 0.1 M KOH	35590	60
30% MeOH + 1% Isoclean in 0.1 M KOH	33140	55
10% EtOH in 0.1 M KOH	31290	52
30% MeOH + 1% polyethylene glycol in 0.1 M KOH	28900	48
30% MeOH + 1% Liquinox in 0.1 M KOH	27790	47

Stock = 5 µg/mL ^{14}C-premafloxacin/glucose

Tetrabutylammonium Hydroxide Extractions

Tetrabutylammonium hydroxide (TBA) in 0.1 M KOH containing methanol or ethanol was the last solvent used to extract

^{14}C-premafloxacin from soil. TBA can form an ion pair with the drug and may effectively reduce some of the soil interactions. A solution containing 30% MeOH 70% 0.1 M KOH in 1% TBA yielded one of the highest recoveries at 65%. The results are listed in Table 5.

Table 5

Scintillation Data From the Basic TBA Extractions

Solvents	DPM	Recovery (%)
100 µL stock + 1 mL blank soil extract (extracted with 1 M KOH)	59759	-----
30% MeOH, 70% 0.1 M KOH with 1% TBA	38740	65
10% EtOH, 80% 0.1 M KOH with 10% TBA	37400	63
30% EtOH, 70% 0.1 M KOH in 1% TBA	33860	57

Stock = 5 µg/mL ^{14}C-premafloxacin/glucose

TBA = tetrabutylammonium hydroxide titrant

Quenching

The scintillation data had a wide range of transformed spectral index of the external standard (tSIE) values (Packard Instrument Co., 1995). These numbers are obtained from the Spectralyzer spectrum

analyzer and are calculated from the spectral distribution of the external standard. They are used as an index of the level of quenching in the samples. In order to determine how these values effected the final DPM counts, several solvents or solvent mixtures were added in 1.0 mL portions to 18 mL of Hionic-Fluor scintillation cocktail. The solvents were combined with 50 µL of the ^{14}C-premafloxacin with glucose stock then the vials were mixed by shaking briefly by hand and analyzed for 2 minutes. The results listed in Table 6 on the following page indicate that the Spectralyzer compensated for the quenching because the DPM results did not vary by more than 3% while the tSIE values ranged from 102 to 372. Relative comparisons can be made between highly quenched and slightly quenched samples according to these results.

Table 6

Scintillation Data From the Quenching Experiment

Solvents	tSIE	DPM
n-butyl chloride	372	53012
0.1 M KOH + 1% TBA	369	52477
sat. NaCl + 1 M HCl	368	53401
80% CH_3CN + 20% EtOH	357	53610
90% CH_3CN + 10% HFBA	357	53032
30% MeOH + 70% 0.1 M KOH 1% TBA	353	51985

Table 6-Continued

4-methyl-2-pentanone	176	51962
chloroform	102	53073

TBA = tetrabutylammonium hydroxide titrant (0.4 M)
HFBA = n-heptafluorobutyric acid (anhydrous)

Solid Phase Extraction Development

Selection of Column Packing Material

I previously extracted non-radiolabeled premafloxacin from swine plasma to develop a method for a toxicokinetic study. This background information was used to begin development of a method to extract the drug from soil by solid phase extraction. C4, C8, C18, cyano, and phenyl silica based bonded phases were examined. Columns containing 50 mg of packing were conditioned with 1 mL of methanol to wet the packing then 1 mL of 0.1% HCl to protonate uncapped silanol groups. A slight vacuum was used to pull the solutions through the columns. The drug was dissolved in 0.1% HCl at a concentration of 1.0 μg/mL and 1 mL was added to the columns. The columns were washed with 1 mL of water and eluted with various solvents. All extracts were analyzed by HPLC with a UV detector set at 300 nm with a mobile phase composed of 35/65 CH_3CN:100 mM citrate buffer at a flow of 0.5 mL/min. The HPLC

column was the Phenomenex column described earlier. Packings were first evaluated by collecting the load effluent and analyzing the effluent using HPLC. The C4 and cyano columns retained the drug poorly while the phenyl and C8 both retained the drug effectively. The C18 packing had the greatest affinity for the drug which made elution difficult. Finding an elution solvent was the next step and the C8 packing was selected for this process.

Columns were prepared with 1 mL of methanol then 1 mL of 0.1% HCl. Premafloxacin stock was applied to the columns followed by 1 mL of water. The columns were eluted and the extracts were analyzed using HPLC/UV. High organic content (45% and above) eluted premafloxacin but effected the adsorption of the drug to the stationary phase during HPLC analysis. This caused peak broadening or another effect which increased the percent recovery to 190% when the elution solvent was 60% acetonitrile and 40% 0.1% HCl. Similar effects were observed by a technician at Keystone Scientific, Inc. in Bellefonte, PA. This company manufactures HPLC columns and have reported peak splitting, band broadening, shift in retention and other effects when solutions with high organic content relative to the mobile phase are injected into the HPLC system.

Acetonitrile was more effective at eluting the drug followed by

methanol. A solution of 40% - 45% acetonitrile produced recoveries closest to 100% under these conditions and when 0.1% HCl was replaced with 1% heptafluorobutyric acid, the effect which caused the high recoveries was diminished. This allowed for an increase in the percent acetonitrile so the final elution solvent was 1 mL of 50/50 CH_3CN:2% HFBA.

Increase in Packing Material

Varian C18 and C8 columns with 1 gram of packing were selected for soil extract clean-up. The columns were conditioned with two column volumes of methanol followed by one column volume of 0.1% HCl. After the columns were drained to the frits, 2 mL of water with 100 µL of the ^{14}C-premafloxacin glucose stock were added and the effluent was collected. The load effluent was collected to determine if the drug was binding to the column. Less than 1% of the drug was detected in the load effluent as shown in Table 7. A wash step with 1 mL of water was used to eliminate residue from the sample loading step. This wash effluent was not analyzed because previous experiments showed that water did not elute the drug from the columns. The columns were drained and eluted with 2 mL of 50/50 CH_3CN:2% HFBA. Scintillation analysis of the C8 eluant resulted in an 89% recovery while the C18

showed 86% recovery. The larger columns were used because more capacity was needed to isolate ^{14}C-premafloxacin from the soil matrix.

Table 7

Scintillation Data From Solid Phase Extractions

Solvents	DPM	Recovery (%)
100 µL stock in 50/50 CH_3CN:2% HFBA	71731	-----
C8 eluant (50/50 CH_3CN:2% HFBA)	63546	89
C18 eluant (50/50 CH_3CN:2% HFBA)	61710	86
C8 load effluent	151	0.2
C18 load effluent	116	0.2

Stock = 5 µg/mL ^{14}C-premafloxacin/glucose
HFBA = n-heptafluorobutyric acid (anhydrous)

Soil Solid Phase Extraction

Column Elution With an Organic Solvent

The C18 packing was tested by extracting a small amount of soil. The previous scintillation data showed that solutions containing sodium dodecyl sulfate (SDS) had some of the highest drug recoveries therefore this compound was initially used to test the C18 packing. The 5 µg/mL

^{14}C-premafloxacin glucose stock (100 µL) was pipetted onto 2 gram portions of soil in three 50 mL polypropylene screw top tubes. Tube 1 received 10 mL of 30% EtOH saturated with SDS. Tubes 2 and 3 received 10 mL of 30% MeOH, 70% 0.1 M KOH with 1% SDS. The tubes were rocked for 2 hours then centrifuged at 2,000 RCF for 8 minutes. The supernatant was dispensed into conditioned C18 columns and the effluent was collected. The results obtained from scintillation counting the effluent indicated that SDS reduced the affinity of ^{14}C-premafloxacin to the C18 packing. A 60% loss occurred when the saturated SDS solution was added to the first column. The solution containing less SDS showed a 12% loss from the second column. When the solution from tube three was added to column three and washed with an equal volume of 0.1% HCl during loading, the drug loss increased to 17%. This portion of the experiment was used to evaluate how washing the column would effect drug retention by comparing column two to column three. The 0.1% HCl eliminated some of the dissolved soil matrix bound to the packing which eluted drug that remained bound to the dark soil matrix. This was a problem because all the columns were washed with 3 mL of water to decrease contaminates in the final extract. The water wash step eliminated most of the soil matrix in addition to more of the drug.

The vacuum was increased to draw the residual water through the

columns then the columns were eluted with 10 mL of 80/20 CH_3CN:EtOH into 20 mL glass scintillation vials. This solvent was used because the drug could be concentrated through evaporation and the solvent would not elute any remaining soil matrix left on the packing. The solvent was evaporated under nitrogen in a water bath at 40-50° C then 18 mL of Hionic - Fluor were added to the vials. The eluant from column two had the highest recovery at 28%. The other two solutions had lower recoveries which were consistent with the increased drug loss during the loading step. Addition of 1 mL of the elution solvent to the scintillation vial from column two increased the DPM count by 25%. The solvent may have reduced glass binding or positively effected the transitions which occur during scintillation. Table 8 lists the results on the following page.

Table 8

Scintillation Data From SDS Soil Solid Phase Extractions

Solvents	DPM	Recovery (%)
100 µL stock in blank soil (30% MeOH,70% 0.1 M KOH 1% SDS)	37878	-----
C18 load effluent (30% EtOH saturated with SDS, 70% 0.1 M KOH with 1% SDS)	22893	60

Table 8-Continued

C18 load effluent (30% MeOH, 70% 0.1 M KOH 1% SDS) with 0.1% HCl wash	6409	17
C18 load effluent (30% MeOH, 70% 0.1 M KOH 1% SDS)	4407	12
C18 eluant (80% CH_3CN 20% EtOH) evaporated to dryness	10591	28

Stock = 5 µg/mL ^{14}C-premafloxacin/glucose
SDS = sodium dodecyl sulfate

Column Elution With Aqueous/Organic Solutions

Solutions containing an aqueous and organic component were used to try to increase the drug recovery. The previous procedure was followed and the tubes were rocked for 24 hours. Column one was eluted with 5 mL of 50/50 CH_3CN:2% HFBA saturated with SDS. The SDS decreased drug binding previously so the compound was added to the elution solvent. Column two had the same eluant without SDS and the third column was eluted with 25/75 CH_3CN:100 mM citrate buffer with 0.5% TEA. Columns one, two and three had recoveries of 17%, 36% and 27% respectively. The addition of SDS in the eluant decreased the recovery under these conditions and increasing the aqueous component did not significantly improve the recovery. All the columns were washed with 3 mL of water prior to the elution step which appeared to elute

drug from the columns with the soil matrix. The remaining soil matrix ended up in the final extracts because the elution solvents had high aqueous content. The extracts were dark and contained impurities which would make analysis by HPLC-RAM difficult.

TBA Soil Extraction

The SDS solutions from the preceding section were not effective so a TBA solution was used to extract 10 grams of soil which had 1 mL of stock added. The amount of soil was increased to determine how this would effect the extraction process since 50 grams of soil would be extracted for the aerobic biodegradation experiment. The soil was extracted with 20 mL of 30% MeOH, 70% 0.1 M KOH with 1% TBA. After rocking for one hour, the soil was re-extracted with an additional 20 mL of the solvent. The centrifuged extracts were added to a conditioned C18 column and the column was not washed with water to decrease drug loss. The column was eluted with 10 mL 80/20 CH_3CN:EtOH followed by 10 mL 90/10 CH_3CN:H_2O. The first 10 mL did not elute much of the soil matrix that was bound to the packing but the second 10 mL which contained 10% water eluted most of the soil matrix. The combined solution was concentrated to 1 mL under nitrogen in a water bath at 40-50° C. The concentrated extract was filtered with a 25 mm 0.45 µm Gelman polysulfone filter which does not bind to the drug.

The extract remained very dark and 39% of the drug was recovered. By using a 10% water solution, the organic solvents could be evaporated while the drug was concentrated to 1 mL without evaporating the solution to dryness. This may have helped reduce loss of drug to the glass but evaporating the solution to a constant volume was difficult and made the assay harder to reproduce. Eliminating the wash step caused an unacceptable amount of soil matrix to appear in the final extract which made the sample unsuitable for HPLC-RAM analysis. This procedure was used to extract the soil used in the aerobic biodegradation experiment even though all the problems with the assay were not solved.

Aerobic Biodegradation Experiment

Preparation of Soil for Incubation

Soil was obtained from a Richland, MI field which met the criteria found in the FDA Technical Assistance Document (TAD) 3.12 regarding pH, organic matter content, etc. The soil must contain sufficient microorganisms in order to mineralize compounds to CO_2. Walter Smolenski tested the soil for viability in another lab by adding glucose to the soil. The microorganisms produced CO_2 from glucose mineralization and the gas was trapped in a sodium hydroxide solution. The CO_2 was quantified by titration with hydrochloric acid and the results from this test showed that addition of glucose to the soil produced more CO_2 than

soil without glucose. The soil was also tested by adding 16 mg of non-radiolabeled premafloxacin to 50 g of soil without glucose. The CO_2 production was monitored for 70 days to determine if the drug would kill microorganisms thus inhibiting CO_2 production. The results indicated that CO_2 production was not diminished by the drug.

TAD 3.12 recommends the extraction of soil which has a dry weight of 50 grams. The soil must also maintain at least 50% of its moisture holding capacity to ensure microorganism viability. The soil was allowed to air dry at room temperature for 6 hours then the soil was placed in a sieve with 2 mm openings in order to remove stones, roots, and other foreign matter. The percent moisture and moisture holding capacity were measured to determine the amount of ^{14}C-premafloxacin solution needed to wet the soil to 60% of its moisture holding capacity. The percent moisture was determined by accurately weighing triplicate 5 gram portions of soil using a balance. The soil was placed in an 110° C oven for 4 hours then the dry soil weight was compared to the original soil weight. The mean percent moisture was 10.24%. No standard deviations were calculated throughout the study due to the preliminary nature of this work. Triplicate funnels containing moist #4 filter paper were filled 3/4 full with soil to determine the moisture holding capacity. The funnels were placed in water with the bottom third under the water line to facilitate capillary absorption of

water by the soil. After the soil absorbed water overnight, the soil was drained for one hour then the soil and funnels were weighed using a balance. The moisture holding capacity was calculated as follows:

Wet = (wet soil)-(funnel)

Moisture Holding Capacity (g water/g dry soil) = (Wet - Dry)/Dry

Dry soil weight was calculated by weighing the soil then subtracting the mean percent moisture from this value. The mean moisture holding capacity was 0.3786 g water/g dry soil.

Since the soil had a percent moisture of 10.24%, the amount of water initially present in 50 g of soil was 5.12 mL. The amount of water needed to moisten the soil to 60% of its moisture holding capacity was calculated as follows:

(50g x 0.3786 mL/g x .60) - 5.12 mL = 6.24 mL

Three samples and two controls were prepared for extraction on six different days. With this amount of soil, the study could be conducted for several weeks if necessary. Soil (55.12 g) was weighed into 500 mL Erlenmeyer flasks then a 5.00 mL aliquot of the ^{14}C-premafloxacin stock was pipetted on the soil samples. The same amount of stock containing glucose without drug was dispensed on the control soils. An additional 1.25 mL of HPLC grade distilled water was

also added to the soil to wet the soil to 60% of its moisture holding capacity. A glass test tube (25 mm x 105 mm) with 10 mL of 2 N sodium hydroxide was placed in each flask to trap any $^{14}CO_2$ which may be evolved during biodegradation. The flasks were capped with rubber stoppers and were aerated three times a week with compressed air passed through an Ascarite II (J.T. Baker) scrubber to remove CO_2. The flasks were stored in the dark at 22° C in a refrigerated incubator.

Incubated Soil Extraction

One original spiked soil sample was extracted after three weeks of incubation to determine if any biodegradation of ^{14}C-premafloxacin could be observed. A solution containing 140 mL of 30% MeOH, 70% 0.1 M KOH with 1% TBA was used to wash the soil into a 200 mL glass Pyrex centrifuge bottle. The bottle was placed in an Eberbach shaker set on "High" for one hour then centrifuged using a Sorval RC3C centrifuge for 15 minutes at 1005 RCF. The dark extract was added to a conditioned C18 column. The column was eluted with 10 mL of 90/10 CH_3CN:H_2O and 5 mL of 80/20 CH_3CN:EtOH. The combined solution was concentrated to 1 mL under nitrogen in a water bath at 40-50° C. The extract was filtered with a 25 mm 0.45 µm polysulfone filter and injected only once into the HPLC-RAM instrument due to the poor condition of the extract. HPLC-RAM analysis of 50 µL showed 97% of the extracted

radiation was parent ^{14}C-premafloxacin producing 13,871 DPM. No peaks were detected in the remaining 3% of the radiation and the chromatogram is shown in Figure 3 on page 32. Scintillation analysis of 50 µL yielded 49,122 DPM. The two DPM values are different because the response ratio entered into the HPLC-RAM was not correct. Since 5 mL of stock were dispensed onto the soil, the extract DPM value was divided by five because the final volume was 1 mL. The extraction efficiency was determined to be 18% by both detection methods as shown in the following calculations:

HPLC-RAM Data

5 µg/mL Stock	Soil Extract
50 µL = 15,586 DPM†	50 µL = 13,871 ÷ 5 = 2,774 DPM

Efficiency = 2,774 ÷ 15,586 x 100 = 18%

† = HPLC-RAM analysis of the stock added to the soil.

Scintillation Data

5 µg/mL Stock	Soil Extract
50 µL = 54,423 DPM‡	50 µL = 49,122 ÷ 5 = 9,824 DPM

Efficiency = 9,824 ÷ 54,423 x 100 = 18%

‡ = Scintillation analysis of the stock added to the soil.

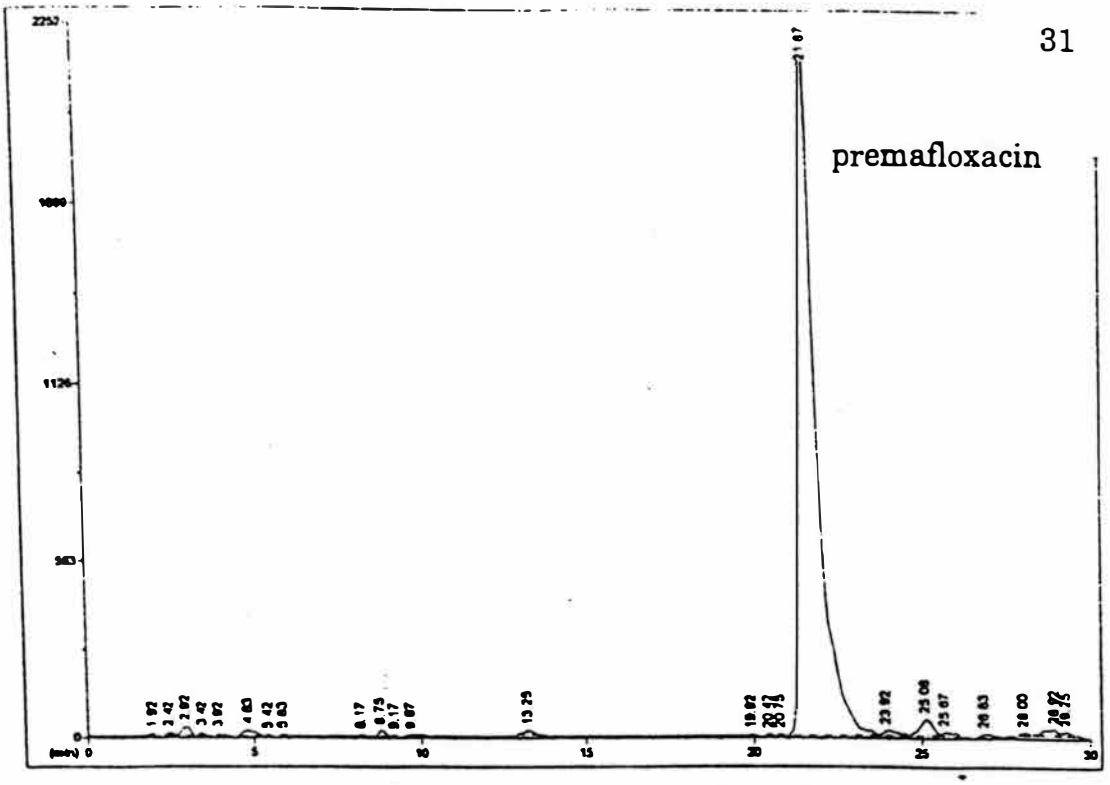
The HPLC-RAM chromatogram of the standard used for this research is shown in Figure 2 on page 31. The HPLC-RAM chromatogram of the 55.12 gram extract after three weeks of incubation

is shown in Figure 3 on page 32. NaOH trapping solutions were also analyzed using scintillation analysis after three and eight weeks of incubation. Less than 1% of the possible $^{14}CO_2$ was detected in each of the two solutions.

Considerations for Future Soil Extractions

If a more comprehensive aerobic biodegradation study is initiated, additional recommendations listed in TAD 3.12 should be considered. The purity of the radiolabeled compound must be 99% so the drug could be purified using a fraction collector followed by freeze drying to concentrate the collected fractions. High purity ensures that radioactive by-products will not be added to the soil prior to degradation.

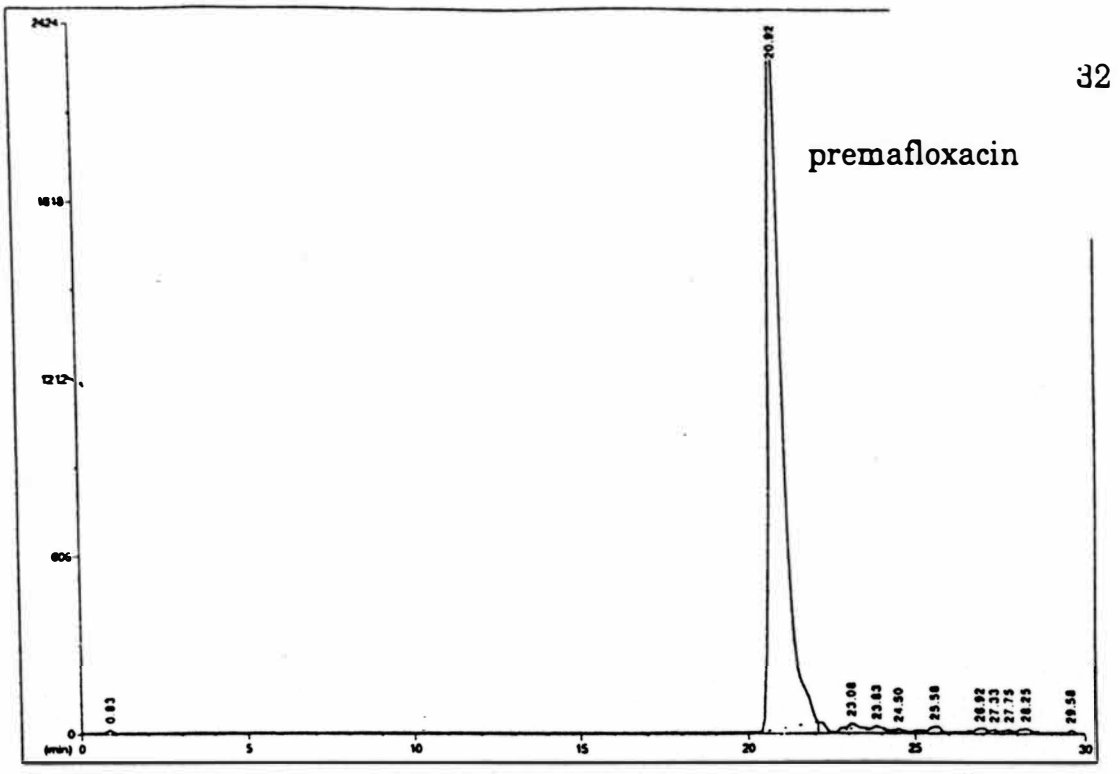
The use of three soil types with different pH ranges is suggested and mass balance can also be estimated by combusting the extracted soil. The test should continue for 64 days or until 50% biodegradation is observed for the test chemical. The test may be terminated if a plateau of CO_2 production is reached. When NaOH trapping solutions were tested after three and eight weeks of incubation, $^{14}CO_2$ production appeared to plateau. The decision to end the study after three weeks of incubation was based on this information combined with the low extraction efficiencies.



Peak Area Report — Channel 1 C-14 DPM

Name	Ret. Time (min.)	Pk#	Area	% Pks
	1.92	1	15	0.09
	2.42	2	30	0.18
	2.92	3	111	0.66
	3.42	4	20	0.12
	3.92	5	10	0.06
	4.83	6	95	0.57
	5.42	7	5	0.03
	5.83	8	10	0.06
	8.17	9	10	0.06
	8.75	10	53	0.32
	9.17	11	5	0.03
	9.67	12	30	0.18
	13.25	13	42	0.25
	19.92	14	10	0.06
	20.42	15	10	0.06
	20.75	16	10	0.06
	21.67	17	15586	93.18
	23.92	18	101	0.60
	25.08	19	284	1.70
	25.67	20	72	0.43
	26.83	21	50	0.30
	28.00	22	23	0.14
	28.92	23	114	0.68
	29.25	24	31	0.19
Total Pks:			16727	

Figure 2. HPLC-RAM Chromatogram of ^{14}C-Premafloxacin Standard.



Peak Area Report — Channel 1 — C-14 DPM

Name	Ret. Time (min.)	Pk#	Area	% Pks
	0.83	1	20	0.14
	20.92	2	13871	96.86
	23.08	3	80	0.56
	23.83	4	48	0.34
	24.50	5	23	0.16
	25.58	6	96	0.67
	26.92	7	58	0.41
	27.33	8	20	0.14
	27.75	9	30	0.21
	28.25	10	59	0.41
	29.58	11	15	0.10
Total Pks:			14320	

Figure 3. HPLC-RAM Chromatogram of the Final Soil Extract After Three Weeks of Incubation. Parent ^{14}C-Premafloxacin Accounted for 97% of the Detected Radiation.

CONCLUSION

Extracting premafloxacin from loam soil proved to be a difficult task. Many solvents were used which had various physical properties. Low extraction efficiencies were observed throughout the study indicating a high binding affinity between ^{14}C-premafloxacin and the soil. This affinity may have decreased the bioavailability of premafloxacin to microorganisms resulting in reduced aerobic biodegradation. When aerobic biodegradation is hindered, CO_2 production will also be low. This was demonstrated by scintillation analysis of the $^{14}CO_2$ trapping solution which showed that less than 1% of the possible $^{14}CO_2$ was produced after three weeks of incubation.

When the soil extract was analyzed using HPLC-RAM, over 90% of the extracted radiation was parent ^{14}C-premafloxacin. The lack of radioactive by-products can indicate reduced aerobic biodegradation. One problem with this data is that radioactive by-products may not have been isolated by the extraction method. The low extraction efficiency is another factor which could inhibit the detection of radioactive by-products.

Although the results from HPLC-RAM analysis are not conclusive, the data from the multiple solvent extractions did prove that

^{14}C-premafloxacin has a high binding affinity for the loam soil used in this study. This data suggests that premafloxacin will become immobilized following contact with loam soil. The probability of the drug dissociating from the soil due to interactions with naturally occurring weak acid, base and saline solutions thus appears to be minimal. Similar conclusions are stated in other fluoroquinolone biodegradation studies involving sarafloxacin (Abbott Laboratories, 1995) and enrofloxacin (Martens, 1996).

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