**UNITED STATES ENVIRONMENTAL PROTECTION AGENCY**
WASHINGTON, D.C. 20460

OFFICE OF
PREVENTION, PESTICIDES
AND TOXIC SUBSTANCES

**MEMORANDUM**

**Date:** July 2, 2003

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**DP Barcode:** D278386

**Petition:** 1F06313

**Citation:** 45405024 Fabian, E.; Grossman, F. (2001) The Metabolism of (carbon-14)-BAS 510 F in Lactating Goats: Final Report: Lab Project Number: 42389: 2000/1017221. Unpublished study prepared by BASF Aktiengesellschaft. 150 p.

45405025 Leibold, E.; Hoffman, H. (2000) (Carbon 14)-BAS 510 F-Absorption, Distribution and Excretion after Repeated Oral Administration in Lactating Goats: Lab Project Number: 02B0426/976039: 2000/1012353. Unpublished study prepared by BASF Aktiengesellschaft. 38 p.

**Sponsor:** BASF Corporation

**Background**

The information contained herein was compiled by the Dynamac Corporation (20440 Century Boulevard, Suite 100, Germantown MD 20874), contractor, under the supervision of RAB2/ HED. This DER has undergone secondary review by PMRA/Canada, and peer review by RAB2, and reflects current HED and Office of Pesticide Programs (OPP) policies.

## Executive Summary

BASF Corporation has submitted a study investigating the metabolism of [$^{14}$C]BAS 510 F in ruminants. The in-life and analytical phases of the study were conducted by BASF Aktiengesellschaft (Limburgerhof, Germany). Two lactating goats were orally dosed with [$^{14}$C]BAS 510 F, uniformly labeled in the diphenyl rings, at an average of 32.43 ppm in the diet once daily for 5 consecutive days. Milk was collected twice daily. The goats were sacrificed 23 hours following the last dose, and samples of kidney, liver, muscle, and fat were collected.

The total radioactive residues (TRR) were 0.010-0.138 ppm in milk collected throughout the dosing period, 0.270 ppm in kidney, 2.593 ppm in liver, 0.036 ppm in fat, and 0.012 ppm in muscle. Residues were highest in liver and lowest in muscle. Radioactivity in milk was highest after dosing and lower by the next milking; however, residues appeared to plateau by the second or third dosing day. Radioactivity in urine, feces, and cage wash collected throughout the dosing period and at sacrifice accounted for 23.7-44.6%, 46.4-64.3%, and 0.9-2.4%, respectively, of the applied radioactivity.

To support the storage conditions and intervals of samples from the study, the extracts of goat matrices analyzed within 3 months of sampling were compared to those analyzed close to the end of the study, revealing no significant changes in the metabolite profile. These data are sufficient to demonstrate the stability of residues in goat matrices for 16.6 months for milk, muscle, fat, and kidney, and 29 months for liver.

Approximately 76-87% of the TRR were extracted from goat milk, muscle, fat, and kidney using solvents; solvent extraction only released ~14% TRR from liver. The parent, BAS 510 F, was detected in all goat matrices, accounting for 2.5-7.9% TRR (0.003-0.129 ppm) in milk and liver, 20.4% TRR (0.002 ppm) in muscle, and 34.6% TRR (0.012 ppm) in fat. Metabolite M510F01 was detected in all goat matrices, at 6.4-26.35% TRR (0.003-0.166 ppm), and metabolite M510F02 was detected in milk (6.4% TRR, 0.002 ppm), muscle (11.9% TRR, 0.001 ppm), and kidney (50.35% TRR, 0.136 ppm). Material balances were ~84-102% TRR.

Subsamples of milk and liver were also subjected to microwave hydrolysis for further characterization/identification of residues. Microwave hydrolysis, using formic or acetic acid, released 88.5% of TRR from milk samples and 100% TRR from liver. In addition to BAS 510 F (7.9% TRR in milk) and M510F01 (19% TRR in milk,6.4% TRR in liver), microwave hydrolysates of liver and milk were found to contain three components that were not identified in any other extracts: M510F49 (11.0% and 7.7% TRR, respectively), M510F51 (2.4% and 12.2% TRR, respectively), and M510F53 (43.6% and 11.2% TRR, respectively). The petitioner concluded that these components corresponded to BAS 510 F, M510F01, and bound or conjugated BAS 510 F, respectively, in milk and liver because microwave hydrolysis of reference compounds indicated that BAS 510 F was converted to M5210F49, M5410F01 was converted to M510F51, and reference compounds in which the pyridine chlorine group had already been substituted with sulfur were converted to M510F52 or M510F53. The petitioner stated that total BAS 510 F residues in milk and liver consisted of the sum of BAS 510 F and M510F49 residues,

total M510F01 residues consisted of the sum of M510F01 and M510F51 residues, and residues of M510F52 or M510F53 corresponded to bound residues in liver or soluble conjugates of glutathione, cysteine, or mercaptic acid in milk.

Based on the results of the study, the petitioner has proposed that BAS 510 F is metabolized in goats through hydroxylation of the diphenyl portion to form M510F01. M510F01 then undergoes glucuronidation to form M510F02. Further hydroxy and thiol substitutions in the diphenyl portion occur. In addition, the chlorine atom on the pyridine ring is substituted by thiol groups in biomolecules.

The ruminant metabolism study is **acceptable**. Although it reflects radiolabeling in the diphenyl ring portion of the molecule only, there was no apparent cleavage of the parent molecule in the subject study (or in the hen metabolism study; see DER of MRID 45405026), and only a very small amount of cleavage was observed in the plant metabolism studies.

**GLP Compliance**

Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were provided. The petitioner stated that the study was conducted in accordance with the GLP regulations established in Germany (Appendix 1 to §19a Section 1, Chemikaliengesetz of 25-July-1994; Official Bulletin/Federal Republic of Germany I 1994, p. 1703), instead of U.S. EPA GLP regulations or PMRA's GLP requirements.

## 1. Materials and Methods

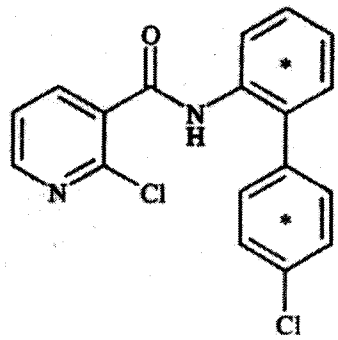
1.1. Substance

Active Ingredient

| | |
|---|---|
| Common Name: | Nicobifen (ISO, proposed) |
| IUPAC Name: | 2-Chloro-N-(4'-chlorobiphenyl-2-yl)nicotinamide |
| CAS Name: | 3-Pyridinecarboxamide, 2-chloro-N-(4'chloro[1,1'-biphenyl]-2-yl)- |
| CAS Number: | 188425-85-6 |
| Company Name: | BAS 510 F |
| Other Synonyms: | BASF Registry No. 300355 |

Purity of Non-labeled Material: >99%

Location of Isotopic Label: Uniformly labeled in both phenyl rings
Radiochemical Purity: 99% (as determined by HPLC)
Specific Activity: 314,000 ppm/μg (labeled compound); 80,890.2 ppm/μg (dosing material), (μCi/mmol not specified)



[Diphenyl-U-$^{14}$C]BAS 510 F

1.2. Test Animals and Site

Species/breed: Goat, "Bunts Deutsche Edelziege"
Age: 15-18 months
Gender: Female
Housing Areas: Stainless steel metabolism cages in a controlled environment (20-24 C and 30-70% Rh).
Diet and Water: Pelleted supplementary goat feed was provided at ≥ 1000 g per goat per day; hay was provided at 500-1000 g per day; tap water was provided *ad libitum*. Feed consumption was measured daily.
Acclimation period: 10 days prior to treatment
Predosing: None

1.3. Dosing

Type(s) of Dosing: Oral by gavage
Dosing Vehicle: [$^{14}$C]BAS 510 F was diluted with non-labeled BAS 510 F in toluene. The mixture was evaporated to dryness and dissolved in 0.5% tylose in aqua bidest, containing 1% Cremophor EL.
Dosing Rate: Nominal of 35 ppm in the diet; actual dose rates were 31.78 and 33.09 ppm (32.43 ppm average); 1.41 and 1.80 mg/kg body weight/day
Number and Duration of Doses: One capsule was administered following the morning milking for 5 consecutive days.

1.4. Sample Collection Procedures

Goats were milked twice daily (a.m. and p.m.). Milk collected in the afternoon was refrigerated overnight and then, with the morning milk, shipped frozen to BASF Agricultural Center (Limburgerhof, Germany). Urine and feces were collected once daily. The treated goats were sacrificed 23 hours following the last dosing. Immediately following sacrifice, the following tissue samples were collected: liver, kidney, kidney fat, intraperitoneal fat, back muscles, leg

muscles, urine, bile, blood, plasma, and GI tract. Cage wash samples were also collected. Samples were frozen (≤-15 C) prior to analysis.

Table 1.4.1. Summary of Storage Conditions

| Goat Matrix | RAC or Extract | Storage Temperature (°C) | Duration (months) | |
|---|---|---|---|---|
| | | | First Analysis | Last Analysis |
| Milk | RAC | -18 | 1.3 | 17.1 |
| Muscle | RAC | | 1.3 | 16.8 |
| Fat | RAC | | 1.7 | 16.8 |
| Kidney | RAC | | 4.3 | 16.8 |
| Liver | RAC | | 0.9 | 29.0 |

The extracts of goat matrices were analyzed within 0.5 months of sample extraction.

To support the storage conditions and intervals of samples from the study, the extracts of goat matrices analyzed within 3 months of sampling were compared to those analyzed close to the end of the study, revealing no significant changes in the metabolite profile. These data are sufficient to demonstrate the stability of residues in goat matrices for 16.6 months for milk, muscle, fat, and kidney, and 29 months for liver.

1.5. Analytical Methods

Total radioactive residues (TRR) were determined at the in-life facility (BASF Department of Toxicology, Ludwigshafen/Rhein, Germany) for milk, urine, and feces. TRR were determined by direct LSC for milk and urine. For feces, the samples were homogenized in water, freeze dried, solubilized (Soluene), and bleached with hydrogen peroxide (24 hours at room temperature) before TRR determination by LSC. TRR determinations for tissue samples and for pooled milk samples were determined at the analytical facility by combustion/LSC or direct LSC. Tissues samples from the two treated goats were pooled prior to TRR determination. The reported LSC limits of quantitation (LOQs; analytical facility) were 0.0007 ppm for milk, and 0.0021-0.0025 ppm for liver, kidney, and muscle. An LOQ for fat was not reported.

Subsamples of milk, muscle, fat, liver, and kidney were subjected to extraction and characterization of residues at the analytical facility. Initially, milk and tissue samples were extracted with methanol (MeOH), and the extracted residues were partitioned with ethyl acetate and water. Aliquots of the organic and aqueous phases were analyzed by LSC to determine the partitioning characteristics of the residue. The MeOH extracts of muscle and kidney were cleaned up by C18 solid phase extraction (SPE) or by filtration for metabolite identification.

Separate samples of milk, liver, and fat were subjected to additional extraction/hydrolysis procedures for metabolite characterization/identification. These procedures are described below.

Milk:

Whey/Protein Separation: A subsample of milk was separated into whey and protein. The sample was adjusted to pH 4.2 using 2 N phosphoric acid and centrifuged. Sodium hydroxide (0.1 N) was added to the solids; after mixing, the pH was adjusted to 4.2 using phosphoric acid, and the mixture was centrifuged. The whey supernatants were combined, acetone was added, and the mixture was cooled (4 C) overnight and filtered. Precipitated lactose was collected by filtration and dissolved in water for LSC determination; the filtrate was concentrated for HPLC analysis. The protein solids remaining following initial hydrolysis were mixed with MeOH and centrifuged. The supernatant was collected, and MeOH:chloroform (1:3, v:v) was added to the remaining residue and centrifuged following mixing. The supernatants were combined, cooled overnight (4 C), filtered, and an aliquot of the filtrate was concentrated for HPLC analysis.

Microwave Treatment: Another subsample of milk was extracted with acetonitrile (ACN) and concentrated. The extract was mixed with acetic acid and subjected to microwave treatment by heating to 170 C over 5 minutes and then maintaining the temperature at 170 C for 0.5 hours. The extract was then mixed with water and cleaned up by C18 SPE. Residues were eluted with MeOH, concentrated, and diluted with MeOH:water (8:2, v:v) for HPLC analysis.

Fat:

ACN/iso-Hexane Extraction: A subsample of fat was extracted with ACN:iso-hexane (1:1, v:v; 3x). The ACN and iso-hexane phases were separately combined, and the iso-hexane phase was extracted with ACN (2x). The residue remaining after ACN:iso-hexane extraction was mixed with 2% ascorbic acid and extracted with ACN:iso-hexane (2x). The remaining solids were extracted with water:MeOH (3:7, v:v). The MeOH extract was combined with the ACN extracts and centrifuged for HPLC analysis.

Liver:

Extraction with Organic Solvents and Protease Digestion: A subsample of liver was extracted with MeOH (3x) and water, and the remaining solids were divided into two subsamples. One subsample was sequentially extracted with ethyl acetate, dichloromethane, and toluene and the extracts were isolated by centrifugation. The second subsample was digested with protease (in 0.1 M TRIS buffer, pH 7.3, 37 C, overnight). The hydrolysate was isolated by centrifugation, and the solids were extracted with MeOH (3x). An additional subsample of liver was directly subjected to protease digestion; however, the resulting mixture could not be separated by centrifugation or cleaned up by SPE.

Trypsin Digestion: A subsample of liver was extracted with MeOH (3x) and water (2x), and the remaining solids were digested with trypsin (0.1 M TRIS buffer, pH 8, 25 C, overnight); the hydrolysate was isolated by centrifugation, and solids were extracted with MeOH (3x).

Acid Hydrolysis: A subsample of liver was refluxed in 5 M HCl in MeOH (1 h) and the hydrolysate was neutralized with NaOH, mixed with MeOH, and cleaned up by C18 SPE; residues were eluted with MeOH. The remaining solids were refluxed with HCl in MeOH a second time.

Sodium Hydroxide Hydrolysis: A subsample of liver was refluxed in 5 M NaOH (1 h), and the resulting hydrolysate was neutralized for HPLC analysis.

Ammonium Hydroxide Hydrolysis: A subsample of liver was refluxed in 5 M ammonium hydroxide (2 h), and the hydrolysate was split into two aliquots: one was cleaned up by C18 SPE, and the other was neutralized with formic acid, centrifuged, and the resulting solids were redissolved in aqueous ammonium hydroxide.
Microwave Treatment: Subsamples of liver were mixed with ACN and acetic or formic acid, heated to 170 C over a period of 5 minutes, and then maintained at 170 C for 0.5 h. The resulting solutions were cleaned up by C18 SPE, concentrated, and diluted with MeOH or MeOH:water (8:2, v:v) for HPLC analysis. The petitioner subjected reference compounds to the same microwave treatments.

Extracts of all matrices were analyzed by HPLC using a Hypersil ENV or YMC C30 column, a UV detector, a radioactivity monitor, and a fraction collector. Gradient mobile phases of 10 mM ammonium formate and ACN or MeOH were used. Residues were identified by co-chromatography with the following reference compounds: BAS 510 F, M510F01, M510F04, M510F05, M510F48, and chlorophenylaminobenzene (M510F62). M510F01, M510F04, M510F05, and M510F48 were obtained from the rat metabolism study (MRID 45404918). M510F02 was also used as a reference standard, although the petitioner did not indicate its source.

LC/MS and LC/MS/MS were used for identification of metabolites isolated from urine and the reaction products of the microwave hydrolysis of reference compounds. Analyses were conducted using electrospray ionization in the positive ion mode. The chromatographic column effluent was split to allow parallel radiodetection and MS analysis.

## 2. Results

Table 2.1. Total Radioactive Residues in Lactating Goats Following Oral Dosing with [Diphenyl-U-$^{14}$C]BAS 510 F at 32.43 ppm for 5 Consecutive Days.

| Matrix | Treatment Day | TRR (ppm) | | |
|---|---|---|---|---|
| | | Goat 1 | Goat 2 | Pooled |
| Milk | Day 1, afternoon | 0.030 | 0.138 | 0.037, 0.039 |
| | Day 1, morning | 0.024 | 0.024 | |
| | Day 2, afternoon | 0.053 | 0.118 | |
| | Day 2, morning | 0.012 | 0.028 | |
| | Day 3, afternoon | 0.045 | 0.112 | |
| | Day 3, morning | 0.010 | 0.026 | |
| | Day 4, afternoon | 0.031 | 0.111 | |
| | Day 4, morning | 0.011 | 0.023 | |
| | Day 5, afternoon | 0.042 | 0.109 | |
| | Day 5, morning | 0.011 | 0.028 | |
| Liver | 23 hours after last dose | -- | -- | 2.593 |
| Kidney | | -- | -- | 0.270 |
| Muscle | | -- | -- | 0.012 |
| Fat | | -- | -- | 0.036 |
| Urine | Days 1-5 | 23.68% of dose | 44.63% of dose | -- |
| Feces | | 64.25% of dose | 46.37% of dose | -- |
| Cage wash | 23 hours after last dose | 0.90% of dose | 2.44% of dose | -- |
| Subtotal, excreta | Entire study | 88.83% of dose | 93.44% of dose | -- |

Table 2.2.1 Extraction, Characterization, and Identification of Radioactive Residues in **Goat Milk (TRR = 0.037 ppm)** Following Oral Dosing with [Diphenyl-U-$^{14}$C]BAS 510 F at 32.43 ppm for 5 Consecutive Days.

| Fraction ID | % TRR | ppm | Residue ID | %TRR | ppm | Comments |
|---|---|---|---|---|---|---|
| MeOH extract | 99.3 | 0.037 | N/A[1] | | | Partitioned with ethyl acetate and water, yielding 52.4% TRR in the organic phase and 36.0% TRR in the aqueous phase. |
| Nonextractable | 5.8 | 0.002 | N/A | | | |
| **Whey/Protein Separation** | | | | | | |
| Whey supernatants | 60.6 | 0.022 | N/A | | | Acetone added to precipitate lactose; precipitate dissolved in water |

| Fraction ID | % TRR | ppm | Residue ID | %TRR | ppm | Comments |
|---|---|---|---|---|---|---|
| Filtrate | 48.8 | 0.018 | M510F02 | 3.7 | 0.001 | Plus 7 unknowns for a total of 35.7% TRR (0.014 ppm); each ≤10.2% TRR (≤0.004 ppm). |
| | | | M510F01 | 9.8 | 0.004 | |
| Dissolved precipitate | 3.0 | 0.001 | N/A | | | |
| Protein fraction - combined organic extracts | 41.0 | 0.015 | N/A | | | Filtered, filtrate concentrated, solids dissolved in water. |
| Filtrate | 33.9 | 0.013 | BAS 510 F | 3.2 | 0.001 | Plus 8 unknowns for a total of 23.0% TRR (0.011 ppm); each ≤8.4% TRR (≤0.003 ppm). |
| | | | M510F02 | 2.7 | 0.001 | |
| | | | M510F01 | 5.1 | 0.002 | |
| Dissolved solids | 1.1 | <0.001 | N/A | | | |
| **Microwave Treatment (TRR = 0.039 ppm)** | | | | | | |
| ACN extract | 88.5 | 0.034 | N/A | | | Subjected to microwave treatment. |
| Hydrolysate | 88.5 | 0.034 | N/A | | | Cleaned up by C18 SPE. |
| C18 MeOH eluate | 65.0 | 0.025 | BAS 510 F | 7.9 | 0.003 | Plus 3 unknowns for a total of 7.0% TRR (0.003 ppm); each ≤4.0% TRR (≤0.002 ppm). |
| | | | M510F51 | 12.2 | 0.005 | |
| | | | M510F01 | 19.0 | 0.007 | |
| | | | M510F53 | 11.2 | 0.004 | |
| | | | M510F49 | 7.7 | 0.003 | |
| Nonextractable | Not reported. | | N/A | | | |

[1] Not analyzed.

Table 2.2.2 Extraction, Characterization, and Identification of Radioactive Residues in **Goat Muscle (TRR = 0.012 ppm)** Following Oral Dosing with [Diphenyl-U-$^{14}$C]BAS 510 F at 32.43 ppm for 5 Consecutive Days.

| Fraction ID | % TRR | ppm | Residue ID | %TRR | ppm | Comments |
|---|---|---|---|---|---|---|
| MeOH extract | 79.7 | 0.010 | N/A[1] | | | Partitioned with ethyl acetate and water, yielding 52.8% TRR in the organic phase and 6.7% TRR in the aqueous phase.<br><br>MeOH extract was also cleaned up by C18 SPE. |
| C18 MeOH eluate | 72.6 | 0.009 | BAS 510 F | 20.4 | 0.002 | Plus 1 unknown at 19.8% TRR (0.002 ppm). |
| | | | M510F02 | 11.9 | 0.001 | |
| | | | M510F01 | 20.6 | 0.003 | |
| Nonextractable | 24.1 | 0.003 | N/A | | | |

[1] Not analyzed.

Table 2.2.3 Extraction, Characterization, and Identification of Radioactive Residues in **Goat Fat (TRR = 0.036 ppm)** Following Oral Dosing with [Diphenyl-U-$^{14}$C]BAS 510 F at 32.43 ppm for 5 Consecutive Days.

| Fraction ID | % TRR | ppm | Residue ID | %TRR | ppm | Comments |
|---|---|---|---|---|---|---|
| MeOH extract | 62.8 | 0.024 | N/A[1] | | | Partitioned with ethyl acetate and water, yielding 52.9% TRR in the organic phase and 2.8% TRR in the aqueous phase. |
| Nonextractable | 24.9 | 0.009 | N/A | | | |
| **ACN/iso-Hexane Extraction** | | | | | | |
| ACN extract | 54.2 | 0.020 | N/A | | | Combined with other ACN and MeOH extracts |
| iso-Hexane extract | Not reported | | N/A | | | Extracted with ACN |
| ACN extract | 3.1 | 0.001 | N/A | | | Combined with other ACN and MeOH extracts |
| iso-Hexane extract | 3.0 | 0.001 | N/A | | | |
| Nonextractable | Not reported | | N/A | | | Extracted with ascorbic acid, ACN, and iso-hexane. |
| ACN extract | 7.5 | 0.003 | N/A | | | Combined with other ACN and MeOH extracts |
| iso-Hexane | 0.9 | <0.001 | N/A | | | |
| Nonextractable | Not reported | | N/A | | | Extracted with ascorbic acid, ACN, and iso-hexane. |
| ACN extract | 2.5 | 0.001 | N/A | | | Combined with other ACN and MeOH extracts |
| iso-Hexane | 0.7 | <0.001 | N/A | | | |
| Nonextractable | Not reported | | N/A | | | Extracted with MeOH:water. |
| MeOH:water extract | 3.6 | 0.001 | N/A | | | Combined with other ACN and MeOH extracts |
| Combined ACN and MeOH extracts | 71.8 | 0.026 | BAS 510 F | 34.6 | 0.012 | Plus 2 unknowns for a total of 10.9% TRR (0.004 ppm); each ≤5.9% TRR (0.002 ppm) |
| | | | M510F01 | 26.3 | 0.009 | |
| Nonextractable | 8.0 | 0.003 | N/A | | | |

[1] Not analyzed.

| Table 2.2.4 Extraction, Characterization, and Identification of Radioactive Residues in **Goat Kidney (TRR = 0.270 ppm)** Following Oral Dosing with [Diphenyl-U-$^{14}$C]BAS 510 F at 32.43 ppm for 5 Consecutive Days. | | | | | | |
|---|---|---|---|---|---|---|
| Fraction ID | % TRR | ppm | Residue ID | %TRR | ppm | Comments |
| MeOH extract | 81.3 | 0.219 | N/A[1] | | | Partitioned with ethyl acetate and water, yielding 61.1% TRR in the organic phase and 13.1% TRR in the aqueous phase.<br><br>MeOH extract was also filtered. |
| Filtrate | 80.8 | 0.218 | BAS 510 F | 2.5 | 0.007 | Plus 8 unknowns for a total of 19.4% TRR (0.052 ppm); each ≤7.6% TRR (≤0.019 ppm) |
| | | | M510F02 | 50.3 | 0.136 | |
| | | | M510F01 | 8.6 | 0.023 | |
| Residue | 0.5 | 0.001 | N/A | | | |
| Nonextractable | 16.3 | 0.044 | N/A | | | |

[1] Not analyzed.

| Table 2.2.5 Extraction, Characterization, and Identification of Radioactive Residues in **Goat Liver (TRR = 2.593 ppm)** Following Oral Dosing with [Diphenyl-U-$^{14}$C]BAS 510 F at 32.43 ppm for 5 Consecutive Days. | | | | | | |
|---|---|---|---|---|---|---|
| Fraction ID | % TRR | ppm | Residue ID | %TRR | ppm | Comments |
| MeOH extract | 16.6 | 0.430 | N/A[1] | | | Partitioned with ethyl acetate and water, yielding 9.6% TRR in the organic phase and 3.1% TRR in the aqueous phase. |
| Nonextractable | 70.7 | 1.833 | N/A | | | |
| **Extraction with Organic Solvents and Protease Digestion** | | | | | | |
| MeOH extract | 14.4 | 0.372 | BAS 510 F | 4.97 | 0.129 | Plus 13 unknowns for a total of 6.52% TRR (0.168 ppm); each ≤1.77% TRR (≤0.046 ppm). |
| | | | M510F01 | 2.86 | 0.074 | Combined with protease digestate and MeOH extract following protease digestion. |
| Water extract | 0.7 | 0.019 | N/A | | | |
| Nonextractable | Not determined | | N/A | | | Calculated to be 86% TRR. Split into two subsamples: one was sequentially extracted with ethyl acetate, dichloromethane, and toluene, which were unsuccessful (<1% TRR released for each extraction); second subsample was digested with protease. |
| Protease digestate | 63.6 | 1.650 | N/A | | | Combined with MeOH extracts. |

| Fraction ID | % TRR | ppm | Residue ID | %TRR | ppm | Comments |
|---|---|---|---|---|---|---|
| Solids | Not reported | | N/A | | | Extracted with MeOH. |
| MeOH extract | 9.7 | 0.250 | N/A | | | Combined with protease digestate and initial MeOH extract. |
| Combined MeOH extracts/ Protease digest | 87.7 | 2.274 | | | | HPLC analysis was conducted, revealing multiple broad peaks. Attempts to isolate these metabolites failed. |
| Solids | 2.5 | 0.064 | N/A | | | |
| **Microwave Treatment (ACN and formic acid)** | | | | | | |
| Hydrolysate | 100 | 2.593 | N/A | | | Cleaned up by C18 SPE. |
| C18 MeOH eluate | 83.0 | 2.152 | BAS 510 F | 5.7 | 0.15 | Plus 5 unknowns for a total of 19.5% TRR (0.50 ppm); each ≤7.2% TRR (≤0.19 ppm). |
| | | | M510F51 | 6.6 | 0.17 | |
| | | | M510F01 | 4.2 | 0.11 | |
| | | | M510F52 | 35.4 | 0.92 | |
| | | | M510F49 | 11.4 | 0.30 | |
| **Microwave Treatment (ACN and acetic acid)** | | | | | | |
| Hydrolysate | 100 | 2.593 | N/A | | | Cleaned up by C18 SPE. |
| C18 MeOH eluate | 63.3 | 1.641 | M510F51 | 2.4 | 0.062 | |
| | | | M510F01 | 6.4 | 0.166 | |
| | | | M510F53 | 43.6 | 1.130 | |
| | | | M510F49 | 11.0 | 0.285 | |

The results of the extraction procedures for liver that were not presented in Table 2.2.5, are summarized below:

Trypsin Digestion: MeOH extraction of liver released 16.2% TRR, trypsin digestion of the nonextractable residues released an additional 31.3% TRR, and MeOH extraction of the remaining solids yielded 13.3% TRR. HPLC analysis of the trypsin digestate revealed one very broad peak.

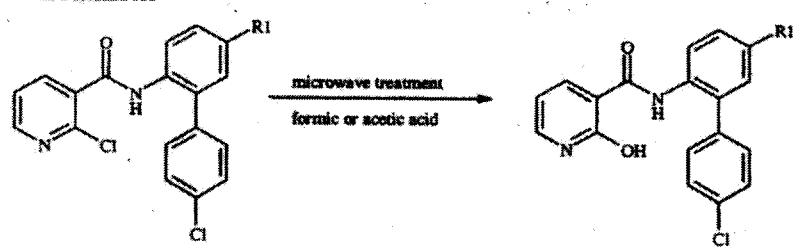
Acid Hydrolysis: The first acid hydrolysis released 32.6% TRR and the second hydrolysis released an additional 32.3% TRR; however, C18 SPE cleanup of the hydrolysates yielded only 18.9% TRR and 15.6% TRR, respectively.

Sodium Hydroxide Hydrolysis: The hydrolysate contained 70.6% TRR; however, HPLC analysis resolved several broad peaks which did not correspond to any of the reference compounds.

Ammonium Hydroxide Hydrolysis: The hydrolysate contained 89.3% TRR; however, attempts to clean up by C18 SPE or to partition the radioactivity into organic solvents were unsuccessful.

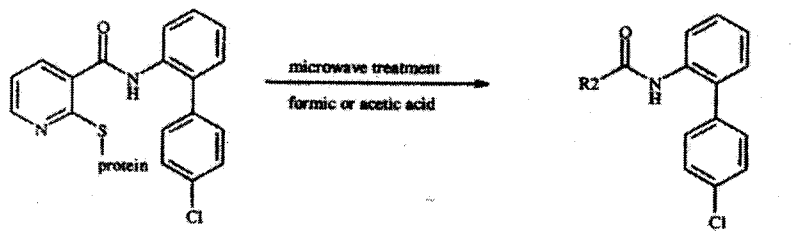
Microwave Treatment: The results of the microwave treatment of reference compounds indicated that microwave treatment caused the substitution of the chlorine atom on the pyridine ring with a hydroxy group, thus converting BAS 510 F to M510F49 and converting M510F01 to M510F51; see diagram below. For reference compounds in which the pyridine chlorine group had already been substituted with sulfur (M510F04, M510F05, and M510F48, obtained from the rat metabolism study), microwave treatment caused the cleavage of the amide bond and acylation of this bond, yielding M510F52 when formic acid was used and M510F53 when acetic acid was used. The petitioner noted that cleavage of the amide bond was not observed when BAS 510 F or M510F01 were subjected to microwave treatment. The compounds obtained from these treatments (M510F49, M510F51, M510F52, and M510F53) were isolated, identified by LC/MS/MS, and used for reference compounds for the analysis of liver microwave hydrolysates. Compounds were identified in milk hydrolysates by comparison of retention times.

Reactions of BAS 510 F reference compounds during microwave treatment




R1 = H; BAS 510 F
R1 = OH; M510F01

R1 = H; M510F49
R1 = OH; M510F51




R2 = H when formic acid used; M510F52
R2 = $CH_3$ when acetic acid used; M510F53

Table 2.3 Summary of Characterization and Identification of Radioactive Residues in Goat Milk and Tissues Following Oral Dosing with [Diphenyl-U-$^{14}$C]BAS 510 F at 32.43 ppm for 5 Consecutive Days.

| Metabolite or Fraction | Milk, Whey/Protein Separation (TRR = 0.037 ppm) | | Milk, Microwave Treatment (TRR = 0.039 ppm) | | Muscle (TRR = 0.012 ppm) | | Fat (TRR = 0.036 ppm) | | Kidney (TRR = 0.270 ppm) | | Liver, Microwave Treatment (ACN/acetic acid) (TRR = 2.593 ppm) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | %TRR | ppm | %TRR | ppm | %TRR | ppm | %TRR | ppm | %TRR | ppm | %TRR | ppm |
| BAS 510 F | 3.2 | 0.001 | 7.9 | 0.003 | 20.4 | 0.002 | 34.6 | 0.012 | 2.5 | 0.007 | --[1] | -- |
| M510F02 | 6.4 | 0.002 | -- | -- | 11.9 | 0.001 | -- | -- | 50.3 | 0.136 | -- | -- |
| M510F01 | 14.9 | 0.006 | 19.0 | 0.007 | 20.6 | 0.003 | 26.3 | 0.009 | 8.6 | 0.023 | 6.4 | 0.166 |
| M510F49[2] | | | 7.7 | 0.003 | -- | -- | -- | -- | -- | -- | 11.0 | 0.285 |
| M510F51[3] | | | 12.2 | 0.005 | -- | -- | -- | -- | -- | -- | 2.4 | 0.062 |
| M510F53[4] | | | 11.2 | 0.004 | -- | -- | -- | -- | -- | -- | 43.6 | 1.130 |
| Unknowns | 58.7 | 0.025 | 7.0 | 0.003 | 19.8 | 0.002 | 10.9 | 0.004 | 19.4 | 0.052 | -- | -- |
| Hexane extracts | -- | -- | -- | -- | -- | -- | 4.6 | 0.002 | -- | -- | -- | -- |
| Retained on C18 SPE | -- | -- | 23.5 | 0.009 | 7.1 | 0.001 | -- | -- | -- | -- | 36.7 | 0.952 |
| Precipitates | 4.1 | 0.002 | -- | -- | -- | -- | -- | -- | 0.5 | 0.001 | -- | -- |
| Total Identified (TI) | 24.5 | 0.009 | 58.0 | 0.022 | 52.9 | 0.006 | 60.9 | 0.021 | 61.4 | 0.166 | 62.4 | 1.643 |
| Total Characterized (TC) | 62.8 | 0.027 | 30.5 | 0.012 | 26.9 | 0.003 | 15.5 | 0.006 | 19.9 | 0.053 | 36.7 | 0.952 |
| Total Extractable (TE) | 87.3 | 0.036 | 88.5 | 0.034 | 79.8 | 0.009 | 76.4 | 0.027 | 81.3 | 0.219 | 99.1 | 2.595 |
| Total Bound (TB) | 0.0 | 0.00 | Not reported | | 24.1 | 0.003 | 8.0 | 0.003 | 16.3 | 0.044 | 0.0 | 0.000 |
| % Mass Balance | 87.3 | | 88.5 | | 103.9 | | 84.4 | | 97.6 | | 99.1 | |

TC = Sum of all unidentified, extractable residues
TE = Sum of TI and TC
% Mass Balance = TE %TRR +TB % TRR

[1] Identified in the methanol extract (4.97% TRR, 0.129 ppm) with organic solvent and protease digestion, and in the C18 SPE eluent (5.7% TRR, 0.15 ppm) with microwave treatment with ACN and formic acid)
[2] Released during microwave hydrolysis; putatively results from hydrolysis of BAS 510 F.
[3] Released during microwave hydrolysis; putatively results from hydrolysis of M510F01.
[4] Released during microwave hydrolysis; putatively results from hydrolysis of bound/conjugated BAS 510 F.

Figure 1. Proposed Metabolic Fate of BAS 510 F in Ruminants (Note that a large portion of the metabolites in Figure 1 were only identified in urine).

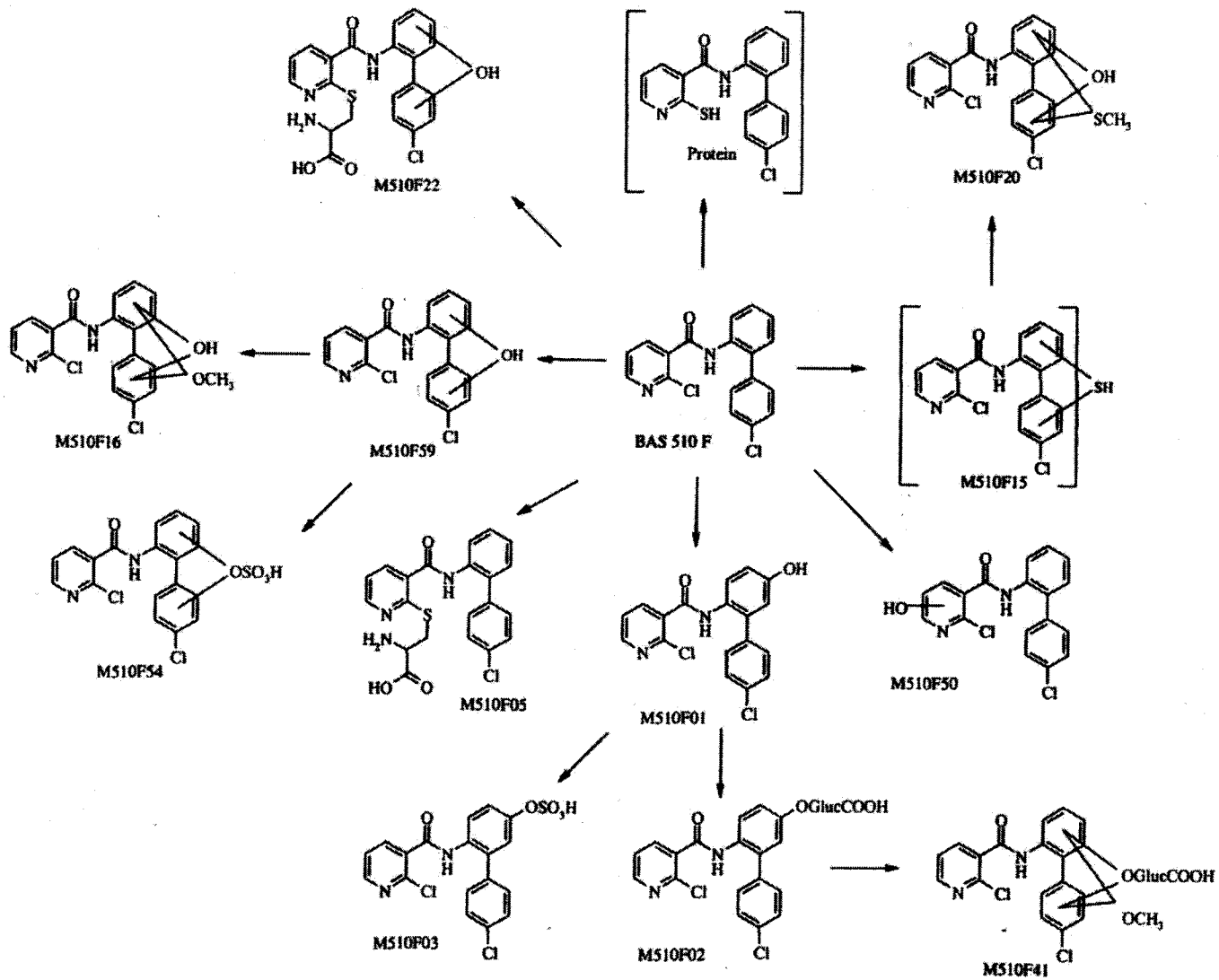


Table 2.4. Metabolites of BAS 510 F in Lactating Goats.

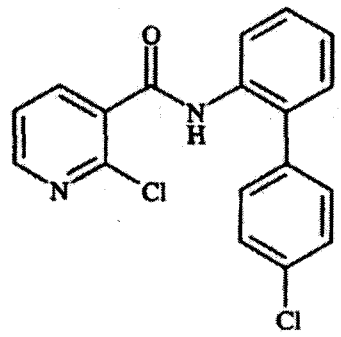
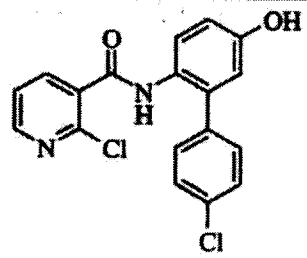
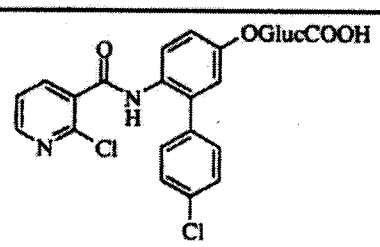
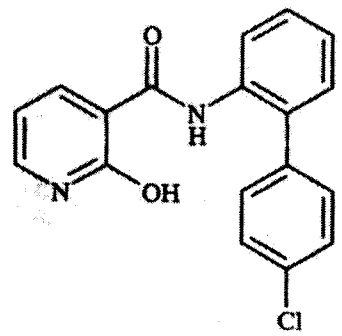
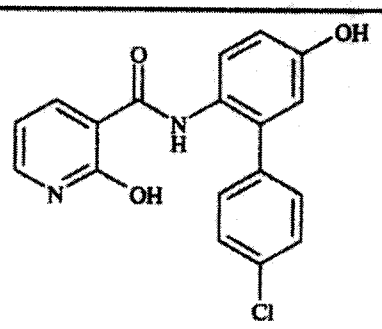
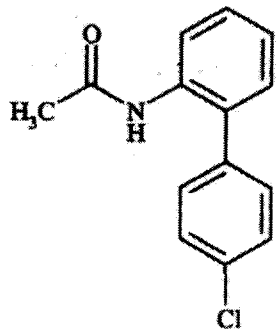
| Metabolite Identifier | Chemical Name | Structure | Comments |
|---|---|---|---|
| BAS 510 F<br><br>(Parent Compound) | 3-Pyridinecarboxamide, 2-chloro-N-(4'chloro[1,1'-diphenyl]-2-yl)- |  | Identified in goat milk, muscle, fat, kidney, and liver |
| M510F01<br><br>(Hydroxy metabolite) | 2-chloro-N-(4'-chloro-5-hydroxy-biphenyl-2-yl) nicotinamide |  | Identified in goat milk, muscle, fat, kidney, and liver |
| M510F02<br><br>(Bound hydroxy metabolite) | glucuronic acid conjugate of M510F01 |  | Identified in goat milk, muscle, and kidney |
| M510F49 | N-(4'-chloro-biphenyl-2-yl)-2-hydroxy-nicotinamide |  | Identified in goat milk and liver; putatively results from microwave hydrolysis of BAS 510 F |

Table 2.4. Metabolites of BAS 510 F in Lactating Goats.

| Metabolite Identifier | Chemical Name | Structure | Comments |
|---|---|---|---|
| M510F51 | N-(4'-chloro-5-hydroxy-biphenyl-2-yl)-2-hydroxy-nicotinamide |  | Identified in goat milk and liver; putatively results from microwave hydrolysis of M510F01 |
| M510F53 | N-(4'-chloro-biphenyl-2-yl)acetamide |  | Identified in goat milk and liver; putatively results from microwave hydrolysis of bound/conjugated BAS 510 F |

## 3. Discussion

### 3.1. Methods

Two lactating goats were orally dosed with [$^{14}$C]BAS 510 F, uniformly labeled in the diphenyl rings, at an average of 32.43 ppm in the diet once daily for 5 consecutive days. Milk was collected twice daily. The goats were sacrificed 23 hours following the last dose, and samples of kidney, liver, muscle, and fat were collected. Total radioactivity was determined using direct LSC for milk samples and combustion/LSC for tissue samples.

Initial extractions with MeOH were performed to demonstrate the efficiency of methanol extraction. MeOH extracted 99.3% TRR from milk, 81.3% TRR from kidney, 79.7% TRR from muscle, 62.8% TRR from fat, and 16.6% TRR from liver. These extracts were not used for metabolite identification, except for muscle and kidney.

Residues in kidney and muscle were adequately extracted with methanol, and fat was extracted with a mixture of acetonitrile, hexane, and ascorbic acid. Liver was subjected to a number of additional extraction/hydrolysis procedures including organic solvent extraction and protease

digestion, trypsin digestion, acid hydrolysis, sodium hydroxide hydrolysis, and ammonium hydroxide hydrolysis. Subsamples of liver and milk residues were also subjected to hydrolysis using formic or acetic acid with microwave heating. Extractable residues were analyzed by HPLC. Material balances were ~84-104% TRR. The methods used to extract and characterize/identify residues adequately elucidated the nature of the residue in the milk and tissues of goats.

3.2. Results

The TRR were 0.010-0.138 ppm in milk collected throughout the dosing period, 0.270 ppm in kidney, 2.593 ppm in liver, 0.036 ppm in fat, and 0.012 ppm in muscle. Residues were highest in liver and lowest in muscle. Radioactivity in milk was highest after dosing and lower by the next milking; however, residues appeared to plateau by the second or third dosing day. Radioactivity in urine, feces, and cage wash collected throughout the dosing period and at sacrifice accounted for 23.7-44.6%, 46.4-64.3%, and 0.9-2.4%, respectively, of the applied radioactivity.

Approximately 76-87% of the TRR was extracted from goat milk, muscle, fat, and kidney using solvents; solvent extraction only released ~14% TRR from liver. The parent, BAS 510 F, was detected in all goat matrices, accounting for 2.5-7.9% TRR (0.003-0.129 ppm) in milk and liver, 20.4% TRR (0.002 ppm) in muscle, and 34.6% TRR (0.012 ppm) in fat. Metabolite M510F01 was detected in all goat matrices, at 6.4-26.35% TRR (0.003-0.166 ppm), and metabolite M510F02 was detected in milk (6.4% TRR, 0.002 ppm), muscle (11.9% TRR, 0.001 ppm), and kidney (50.35% TRR, 0.136 ppm). The petitioner concluded, based on the results of ammonium hydroxide hydrolysis of liver, that nonextractable residues in liver, which accounted for ~71% TRR following methanol extraction, were bound to liver proteins. This was supported by the rat metabolism study, which had shown conjugation of BAS 510 F with glutathione through substitution of the pyridine chlorine atom with the thiol groups of cysteine.

Microwave hydrolysis, using formic or acetic acid, released 88.5% of TRR from milk samples and 100% TRR from liver. In addition to BAS 510 F (7.9% TRR in milk) and M510F01 (19% TRR in milk,6.4% TRR in liver), microwave hydrolysates of liver and milk were found to contain three components that were not identified in any other extracts: M510F49 (11.0% and 7.7% TRR, respectively), M510F51 (2.4% and 12.2% TRR, respectively), and M510F53 (43.6% and 11.2% TRR, respectively). The petitioner concluded that these components corresponded to BAS 510 F, M510F01, and bound or conjugated BAS 510 F, respectively, in milk and liver because microwave hydrolysis of reference compounds indicated that BAS 510 F was converted to M5210F49, M5410F01 was converted to M510F51, and reference compounds in which the pyridine chlorine group had already been substituted with sulfur were converted to M510F52 and M510F53. The petitioner stated that total BAS 510 F residues in milk and liver consisted of the sum of BAS 510 F and M510F49 residues, total M510F01 residues consisted of the sum of M510F01 and M510F51 residues, and residues of M510F52 or M510F53 corresponded to bound residues in liver or soluble conjugates of glutathione, cysteine, or mercaptic acid in milk.

## 4. Deficiencies

No deficiencies were identified.

## 5. References

None.