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DATA EVALUATION RECORD

STUDY TYPE: Metabolism - Rat; OPPTS 870.7485; OECD 417.

PC CODE: 107104

DP BARCODE: 341456

TEST MATERIAL (RADIOCHEMICALPURITY: [^{14}C] 2-methyl-4-isothiazolin-3-one (99.08%)

SYNONYMS: [4,5-^{14}C]-RH-573), [^{14}C]-RH-573, Kordek 573T

CITATION: Kim-Kang, H., L. Cai, and D. Wu (2005). Metabolism and pharmacokinetics of ^{14}C-RH-573 in the rat. XenoBiotic Laboratories, Inc., Plainsboro, NJ. XBL Report No. RPT01057, June 13, 2005. MRID 47154009. Unpublished.

SPONSOR: Rohm and Haas Company, Spring House, PA.

EXECUTIVE SUMMARY: In a 96-hour metabolism study (MRID 47154009), ^{14}C-RH-573 (Lot #724.0501, 99.08% radiochemical purity) dissolved in water was administered by gavage to rats at a dose of 5 or 50 mg/kg (4 animals/sex/dose for excretion and metabolite characterization studies; 3 animals/sex/dose for blood and plasma pharmacokinetic studies). A control group was not dosed and was sacrificed at study initiation (1 rat/sex).

In the low-dose group, a mean of 94.02-96.00% of the radioactive dose was recovered by 96 hours post-dosing, with 56.04-65.21% found in the urine, 20.65-29.11% in the feces, 2.49-3.63% in selected tissues, and 4.52-8.37% in cage rinse. In the high-dose group, a mean of 91.71-92.52% of the radioactive dose was recovered, with 47.13-49.59% found in the urine, 33.51-37.39% in the feces, 1.88-2.17% in selected tissues, and 6.12-6.45% in cage rinse. Blood had the highest percent radioactivity in tissues (mean of 1.73-2.49% for the low-dose group, 1.30-1.45% for the high-dose group). Each of the other tissues contained less than 1% of the dose. Most of the administered dose (mean of 80.38-87.29%) was eliminated within 24 hours post-dosing.

Approximately 23 metabolites of RH-573 were detected. The main metabolites in the urine, designated as M1 and M12, contained 20.8 6-23-29% and 9.73-22.67% of the administered dose, respectively. M1 was identified as N-methyl-malonamic acid. M12 was identified as a 3-mercapturic acid conjugate of 3-thiomethyl-N-methyl-propanamide. Urinary metabolites M3 and M9 comprised 4.33-5.63% and 2.42-6.18% of the dose, respectively. The main metabolite in the feces, designated as M2, consisted of four compounds and contained 12.20-25.78% of the administered dose. Each of the other metabolites comprised <3% of the administered dose. Structures were also proposed for two of the components of M2, M3, M9 (composed of two isomers) and seven minor metabolites. A metabolic pathway was proposed for RH-573 in the rat.

In the low-dose group, T_{max} in blood and plasma was reached for both sexes at one hour post-dosing. In the high-dose group, T_{max} was reached at 1.7 hours in males and at 3 hours in females. The elimination half-lives of ^{14}C-label from plasma ($T_{1/2}$ initial) were rapid and ranged from 3 to 6 hours.

This metabolism study in the rat is classified **ACCEPTABLE - NONGUIDELINE** and does not satisfy the guideline requirement for a metabolism study [OPPTS 870.7485, OECD 417] in the rat. The study is upgradable if justification is provided for not collecting expired air samples, or urine samples at 6 and 12 hours post-dosing and/or if these requirements are waived. **The absence of these data does not significantly affect the results of the study.**

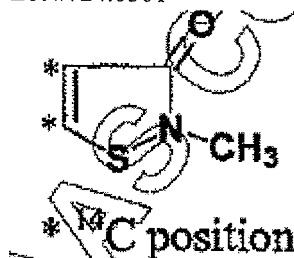
COMPLIANCE: Signed and dated GLP, Quality Assurance, and No Data Confidentiality statements were provided. The study was performed in accordance with 40 CFR Part 160, with the exception that the NMR analyses were not conducted under a GLP program.

I. MATERIALS AND METHODS:

1. **Test compound:**

Radiolabelled test material: [^{14}C]- 2-methyl-4-isothiazolin-3-one

- **Radiochemical purity:** 99.08% (method of determination not provided)
- **Specific activity:** 25.20 mCi/g
- **Lot/batch #:** Lot #724.0501
- **Structure:**



Non-Radiolabelled test material: 2-methyl-4-isothiazolin-3-one

- **Description:** Colorless to brown clear liquid
- **Lot/batch #:** Lot #800IJ123 (TD #01-119)
- **Purity:** 51.4%
- **Contaminants:** None reported
- **CAS # of TGAI:** Not provided

2. **Vehicle and/or positive control:** The dosing solution was prepared in NANOPure™ water.

3. **Test animals:**

- **Species:** Rat
- **Strain:** Sprague-Dawley
- **Age/weight at study initiation:** Adult (7-10 weeks); ~214-251 grams
- **Source:** Charles River Laboratories, Inc. (Raleigh NC; Kingston, NY; Portage, MI)
- **Housing:** Individual stainless steel metabolism cages
- **Diet:** Certified Purina Rodent Chow® #5002 in powder form, *ad libitum* except 4 hr post-dose; feed analyzed by manufacturer for nutritional contents and contamination
- **Water:** Tap water, *ad libitum;* water samples analyzed by International Hydronics Corp. (Rocky Hill, NJ) for selected organophosphates and chlorinated hydrocarbons
- **Environmental conditions:**
  - **Temperature:** 19-25°C
  - **Humidity:** 50±20%
  - **Air changes:** not provided
  - **Photoperiod:** ~12 hrs dark/12 hrs light
- **Acclimation period:** 5 days in polycarbonate cages (≤5/cage); fed Certified Purina Rodent Chow® #5002 in pellet form, *ad libitum*
 ~24 hr acclimation in study cages; fasted overnight prior to dosing; fresh water available *ad libitum* during acclimation through a bottle dispenser attached to cage

4. **Preparation of dosing solutions:** The low- and high-dose solutions (target concentration of 0.5 mg/mL and 5 mg/mL, respectively) were prepared by weighing an appropriate amount of ^{14}C-RH-573 into the dose bottle, adding a sufficient amount of NANOPure™ water, and mixing until dissolved. Pre- and post-dose concentrations were assayed by weighing duplicate aliquots of 100 µL of the dosing solution into 5 mL volumetric flasks and diluting to 5 mL with water. After mixing thoroughly, duplicate aliquots (100 µL) were analyzed with a liquid scintillation spectrometer (LSS). The dose solution for Groups A and B was assayed by taking triplicate aliquots at pre-dose and duplicate aliquots post-dose. The stability of the test substance in the dose formulation was confirmed using HPLC.

B. STUDY DESIGN AND METHODS:

1. **Group arrangements:** Animals were identified upon receipt by a unique identification number on an ear tag and marked on the tail with indelible ink. Animals were assigned to the test groups noted in Table 1 (method of assignment not specified). Animals in Groups A, B, C, and D were sacrificed at 96 hours; one animal not dosed in Group E was sacrificed at Time 0, the 3 others were dosed and sacrificed at T_{max} = 1 hour.

TABLE 1: Dosing groups for pharmacokinetic studies for ^{14}C-RH-573

Test group	Dose of labeled material (mg/kg)	Dose volume (mL/kg)	No. of rats/sex	Samples Collected
A (low dose)	5	~10	4	Urine, feces, tissues
B (low dose)	5	~10	3	Blood/plasma
C (high dose)	50	~10	4	Urine, feces, tissues
D (high dose)	50	~10	3	Blood/plasma
E (low dose)	5 0	~10 0	3 1	Excreta/Tissues

2. **Dosing and sample collection:** Animals received a single dose by oral gavage at the nominal dose based on its body weight prior to dosing. The actual administered dose was determined by weighing the syringe when loaded and after delivery of the dose.

a. Pharmacokinetic studies:

Sample Collection: Samples were collected from the test groups as indicated above in Table 1.

Urine and feces were collected from Group A and Group C pre-dose and at post-dose intervals of 0-24, 24-48, 48-72, and 72-96 hours. Urine samples were collected into tared cups and freeze-trapped using dry ice. Feces samples were collected at room temperature from the cage screen, then weighed and stored frozen. Cages were rinsed with NANOPure® water at 24, 48, and 72 hours post-dose. At the end of the study, cages were washed with IPA/water (1:1). Cage rinse samples were collected in tared containers. Urine, feces, and cage rinse were collected from Group E at the time of sacrifice.

Blood (~0.4 mL) was collected from the tail vein of Group B and Group D animals at 1, 3, 6, 14, 48, and 72 hours post-dose. At the time of sacrifice, animals from Groups A

through D were anesthetized with carbon dioxide and blood (~5 mL) was collected by cardiac puncture in heparinized collection vessels.

At sacrifice, the following tissues were collected from Groups A, C, and E: liver, fat, kidneys, bone marrow (femur), heart, lungs, brain, testes, ovaries, muscle (hind leg), spleen, adrenals, thyroids, and remaining carcass. Tissues were stored at approximately -20°C until analysis.

Sample Preparation: Blood was mixed manually by inverting the collection tubes. Duplicate aliquots (~0.07-0.1 grams) were weighed out for combustion. The remaining blood was centrifuged (2500 rpm, 10 min, 4°C) to obtain plasma. Duplicate subsamples of plasma were weighed (~0.07-0.1 grams, as allowable).

Urine and cage rinse samples (~0.1 mL for urine, ~1 mL for cage rinse) were mixed thoroughly. Duplicate aliquots were weighed into scintillation vials and mixed with scintillation cocktail. Feces were homogenized with ~3-5x (w/v) NANOPure® water and triplicate aliquots of homogenate (equivalent to ~100 mg fresh feces weight) were combusted in a Harvey Biological Sample Oxidizer. Smaller samples were analyzed if expected to contain very high radioactivity.

Tissues were homogenized with 2x (w/v) NANOPure® water. Duplicate aliquots (equivalent to ~100 mg fresh tissue weight, as allowable) were combusted in a Harvey Biological Sample Oxidizer.

Sample Analysis: Radioactivity in plasma, urine, cage rinse, and feces extract samples was counted directly by a liquid scintillation counter (LSC). Total radioactive residue (TRR) levels in tissues, feces, or post-extraction solid (PES) samples were determined by combusting aliquots of homogenized samples in a Harvey OX-500 or OX-300 Biological Sample Oxidizer; the evolved $^{14}CO_2$ was counted in 15 mL of Harvey Scintillation Carbon-14 Cocktail. Samples analyzed for excretion data were counted in duplicate (direct counting) or triplicate (combustion analysis). Samples for metabolite profiling (See following subsection) were usually limited to a single or duplicate analysis. Samples were analyzed with a Beckman LS 6000IC, LS 6000LL, or LS 6000TA. Samples were counted for 10 min or until the 2-sigma error was less than or equal to 2%. The counting time for HPLC fractions was 2 min. Quench correction was performed using an external standard method. Oxidizer efficiencies were validated by combusting a known amount of ^{14}C-mannitol.

For TRR levels, the scintillation spectrometer was set to zero background. Control and treated samples were counted for each matrix, and counts per minute (cpm) automatically converted to disintegrations per minute (dpm). The dpm from the control sample was subtracted from the treated sample and the net dpm per aliquot used for subsequent calculations.

b. **Metabolite characterization studies:** Samples of urine collected at 0-24 hr post-dose, and feces collected at 0-24 hr and 24-48 hr post-dose intervals were analyzed for metabolic profiling. Urine and fecal samples were pooled proportionally according to

gender, treatment, and/or time interval. Reverse-phase HPLC (RP-HPLC) was used to obtain radioprofiles of pooled samples. Four pooled urine samples and eight pooled feces samples were analyzed by RP-HPLC. Metabolites were designated according to their retention times. Further characterization and identification of some metabolites were obtained by LC/MS and/or LC/MS/MS analysis.

Prior to HPLC analysis, urine samples were filtered with Waters Alliance Filtration Manifold filter, GHP 0.45 μm.

Prior to HPLC analysis, the pooled feces samples were extracted and concentrated. The feces samples were extracted using CH_3OH (~5x v/w); following centrifugation the precipitate was mixed with water and CH_3OH (1:9, 5x v/w), sonicated, and centrifuged. The resulting supernatant was combined with the first supernatant, the volume adjusted to 25 mL with methanol, and aliquots taken for LSC. Triplicate aliquots (~50 mg) of PES were combusted and radioassayed by LSC. The same procedure was followed with a pre-dose feces sample fortified with ^{14}C-RH-573 to evaluate the extractability and extraction stability of the parent compound.

HPLC was conducted under one of three conditions (Condition 2, 3, or 4). The initial metabolite radioprofiles for urine and feces extracts were determined by a Waters® 2695 HPLC System coupled with a Packed Radiomatic Series 500TR Flow Scintillation Analyzer (Condition 2). Condition 3 was used for metabolite isolation and Condition 4 was used for the analysis of the M2 isolate.

Because of a large amount of co-eluting matrices, direct LC/MS analyses of the urine samples and feces extracts could not provide definitive molecular ions for most of the metabolites. One urine sample and one feces extract were subjected to preparative HPLC (Condition 2) and the eluates collected every 15 sec into 2-mL, 96-well fraction collection plates. Multiple collections were made for each sample to obtain a sufficient quantity of metabolites for subsequent HPLC or LC/MS and/or LC/MS/MS analyses. LC/MS was conducted under one of seven conditions (Condition 1,2, 3, 4, 5, 6, or 7).

3. **Statistics:**

Pharmacokinetic Analysis: Pharmacokinetic parameters were estimated by non-compartmental techniques using validated WinNonlin software (Pharsight, version 3.2). The maximum plasma concentration (C_{max}) was calculated using the data of individual rats. The area under the plasma concentration-time curve over time was calculated using the linear trapezoidal method. The first-order terminal elimination rate constant was determined by linear regression of the terminal phase of the log phase of the log plasma concentration curve. The apparent terminal half-life was calculated as $t_{1/2}$ 0 0.693/K_{el}. The area under the curve from time zero to time infinity was calculated as $AUV_{0\text{-}inf} = AUC_{0\text{-}t} + C_i/K_{ci}$. The plasma concentration versus time curve follows a biphasic elimination. The initial rate of elimination (faster phase) was estimated using data points from 1 to 24 hours. The initial elimination half-life was determined from this parameter.

Calculations: Calculations were performed using an electronic calculator or an IBM-

compatible PC with Microsoft® Excel software.

The percent dose recovered in each matrix at each interval was calculated as the total dpm in matrix/dpm dosed x 100. The percentages of doses recovered in blood, fat, and muscle were extrapolated using the following factors: 20.4 grams blood, 10 grams fat, and 125 grams muscle per 250 grams body weight.

For metabolite profiling, radioactivity in pooled urine and feces samples was expressed as percent of administered dose. The percent region of interest (ROI) in each HPLC chromatogram was calculated as the dpm in a peak region/total dpm in interested peak regions x 100. The percent dose, recovered as RH-573 or metabolite in pooled urine was calculated as the (%ROI x % dose in the sample)/100. The percent dose in pooled feces extracts was calculated as the (% fractional distribution x % dose in the pooled feces sample)/100. The percent dose recovered as RH-573/metabolite in feces was calculated as (%ROI x % dose in the extracts)/100.

II. RESULTS:

A. PHARMACOKINETIC STUDIES:

1. **Preliminary experiments:** According to the study protocol, the dose levels used in the study were chosen based on previous toxicological studies using the test compound. No further information regarding these studies or their results was provided.

2. **Absorption:** Absorption was not directly addressed in the report.

3. **Tissue distribution:** The percent of the radioactive dose in selected tissues at 96 hours post-dosing is summarized for Group A (low dose) and Group C (high dose) in Table 2. The mean total percent of the radioactive dose was 2.49-3.63% for the low-dose group and 1.88-2.17% for the high-dose group. The mean percent of the administered dose in individual tissues was <0.01-2.49% for the low dose group and <0.01-1.45% for the high-dose group. Blood had the highest percent radioactivity (mean of 1.73-2.49% for the low-dose group, mean of 1.30-1.45% for the high-dose group). The mean percent radioactivity in each of the other analyzed tissues was <1% of the administered dose.

TABLE 2: Distribution of radioactivity in rat tissues/organs 96 hours after oral administration of ^{14}C-RH-573[a]

Tissue/organ	Percent of radioactive dose administered (mean±SD)			
	Low dose (Group A)		High dose (Group C)	
	Male (n=4)	Female (n=4)	Male (n=4)	Female (n=4)
Adrenals	<0.01±NA	<0.01±NA	<0.01±NA	<0.01±NA
Blood	2.49±0.19	1.73±0.17	1.45±0.19	1.30±0.11
Bone marrow	<0.01±NA	<0.01±NA	<0.01±NA	<0.01±NA
Brain	<0.01±NA	<0.01±NA	<0.01±NA	<0.01±NA
Fat	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.01
Heart	0.01±0.01	0.02±0.01	0.01±0.00	0.01±0.01
Kidneys	0.03±0.01	0.03±0.01	0.03±0.00	0.02±0.00

TABLE 2: Distribution of radioactivity in rat tissues/organs 96 hours after oral administration of ^{14}C-RH-573[a]

Tissue/organ	Percent of radioactive dose administered (mean±SD)			
	Low dose (Group A)		High dose (Group C)	
	Male (n=4)	Female (n=4)	Male (n=4)	Female (n=4)
Liver	0.23±0.03	0.11±0.02	0.13±0.03	0.09±0.01
Lungs	0.03±0.01	0.03±0.01	0.02±0.01	0.02±0.00
Muscle	0.80±0.18	0.56±0.03	0.50±0.07	0.42±0.06
Plasma	<0.01±NA	<0.01±NA	<0.01±NA	<0.01±NA
Spleen	0.01±0.00	0.01±0.01	0.01±0.00	0.01±0.00
Thyroid	<0.01±NA	<0.01±NA	<0.01±NA	<0.01±NA
Testes	0.01±0.00	NA	<0.01±NA	NA
Ovaries	NA	<0.01±NA	NA	<0.01±NA
Total	**3.63±0.36**	**2.49±0.14**	**2.17±0.26**	**1.88±0.16**

NA – Not applicable

[a] Data were obtained from pages 54 and 55 (Table V and Table VI) of the study report.

The mean radioactivity concentrations in selected tissues (µg equivalent/gram tissue) at one hour post-dose (T_{max}, see Section IIA5) in the low-dose group (Group E) are summarized in Table 3. The mean concentration was highest in the kidneys (13.364-15.528 (µg equiv/gram), followed by the thyroid (4.956-6.640 µg equiv/gram), liver (3.788-3.796 (µg equiv/gram), and blood (3.438-3.649 µg equiv/gram).

TABLE 3: Distribution of radioactivity in rat tissues/organs in the low-dose group (Group E) one hour (T_{max}) after oral administration of ^{14}C-RH-573[a]

Tissue/organ	µg equivalents/gram (mean±SD)	
	Male (n=3)	Female (n=3)
Adrenals	1.560±0.04	2.112±0.29
Blood	3.438±0.18	3.649±0.59
Bone marrow	1.824±0.09	2.046±0.17
Brain	0.735±0.05	0.774±0.07
Fat	0.485±0.05	0.630±0.33
Heart	1.958±0.31	1.942±0.19
Kidney	15.528±1.76	13.364±8.57
Liver	3.796±0.51	3.788±0.93
Lungs	2.675±0.34	2.815±0.23
Muscle	0.993±0.02	0.944±0.06
Plasma	2.851±0.14	3.120±0.27
Spleen	1.822±0.18	1.930±0.19
Thyroid	4.956±0.43	6.640±1.58
Testes	0.958±0.07	NA
Ovaries	NA	1.943±0.17

NA – Not applicable

[a] Data were obtained from page 58 (Table IX) of the study report.

4. **Excretion:** The excreted dose was considered to be the sum of the radioactivity in the urine, feces, and cage rinse. The recovery of radioactivity in excreta and cage rinse is summarized in Table 4. By 96 hours post-dosing, a mean of 90.37-93.51% of the administered dose was

excreted by the low-dose group; 89.54-90.64% was excreted by the high-dose group. The highest percentage of radioactivity was found in the urine (56.04-65.21% for the low-dose group, 47.13-49.59% for the high-dose group), followed by the feces (20.65-29.11% for the low-dose group, 33.51-37.39% for the high-dose group). Most of the administered dose was eliminated within the first 24 hours (80.38-87.29% for the low-dose group; 83.86-84.23% for the high-dose group).

TABLE 4: Recovery of radioactivity in excreta and cage rinse of rats after oral administration of ^{14}C-RH-573[a]

Matrix/interval	Percent of radioactive dose administered (mean±SD)			
	Low dose (Group A)		High dose (Group C)	
	Male (n=4)	Female (n=4)	Male (n=4)	Female (n=4)
Urine				
0-24 hr	64.11±3.16	53.73±9.24	48.20±4.39	45.97±2.56
24-48 hr	0.69±0.34	1.08±0.68	0.80±0.28	0.73±0.33
48-72 hr	0.27±0.20	0.94±0.49	0.40±0.14	0.26±0.12
72-96 hr	0.14±0.03	0.28±0.26	0.19±0.01	0.17±0.07
Subtotal	**65.21±2.89**	**56.04±7.82**	**49.59±4.14**	**47.13±2.93**
Feces				
0-24 hr	19.58±3.43	22.48±8.82	31.64±1.72	33.71±4.16
24-48 hr	0.84±0.72	5.88±6.30	1.40±0.59	3.16±1.41
48-72 hr	0.19±0.19	0.59±0.65	0.26±0.07	0.38±0.13
72-96 hr	0.04±0.01	0.17±0.13	0.21±0.17	0.14±0.13
Subtotal	**20.65±2.72**	**29.11±2.53**	**33.51±1.54**	**37.39±3.16**
Cage Rinse				
0-24 hr	3.60±2.57	4.17±2.87	4.02±1.93	4.55±2.41
24-48 hr	0.15±0.10	1.03±0.65	0.71±0.48	0.28±0.16
48-72 hr	0.19±0.18	0.72±0.37	0.31±0.17	0.27±0.24
72-96 hr	0.58±0.43	2.45±2.14	1.41±1.04	1.02±0.73
Subtotal	**4.52±2.50**	**8.37±4.95**	**6.45±1.71**	**6.12±2.93**
Total	**90.37±3.72**	**93.51±4.55**	**89.54±2.10**	**90.64±0.94**

[a] Data were obtained from page 52 (Table III) of the study report.

Table 5 summarizes the percent dose recovery from excreta (urine, feces), cage rinse, and selected tissues 96 hours post-dosing for Group A and Group C. The total percent recovery of the administered dose in these matrices ranged from 94.02-96.00% for the low-dose group, and from 91.71-92.52% for the high-dose group.

TABLE 5: Summary of percent dose recovery from excreta, tissues, and cage rinse 96 hours after oral administration of ^{14}C-RH-573[a]

Matrix	Percent of radioactive dose administered (mean±SD)			
	Low dose (Group A)		High dose (Group C)	
	Male (n=4)	Female (n=4)	Male (n=4)	Female (n=4)
Urine	65.21±2.89	56.04±7.82	49.59±4.14	47.13±2.93
Feces	20.65±2.72	29.11±2.53	33.51±1.54	37.39±3.16
Cage rinse	4.52±2.50	8.37±4.95	6.45±1.71	6.12±2.93
Tissues	3.63±0.36	2.49±0.14	2.17±0.26	1.88±0.16
Total	**94.02±NC**	**96.00±NC**	**91.71±NC**	**92.52±NC**

NC – A standard deviation was not calculated for the mean of the total.
[a] Data were obtained from pages 52, 54 ,55, and 59 (Table II, Table V, Table VI, and Table X) of the study report.

5. **Plasma and Blood Concentrations and Pharmacokinetics:** Blood and plasma concentrations of total radioactivity of ^{14}C-RH-573 are summarized for Group B and Group D in Table 6 and Table 7, respectively.

TABLE 6: Total radioactivity concentration of ^{14}C-RH-573 in rat blood[a]

Time Point (hr)	µg equivalents/gram (mean±SD)			
	Low dose (Group B)		High dose (Group D)	
	Male (n=3)	Female (n=3)	Male (n=3)	Female (n=3)
1	3.384±0.49	3.038±0.36	21.127±3.43	17.741±2.35
3	2.622±0.28	1.893±0.06	18.561±1.07	20.846±4.81
6	1.897±0.13	1.381±0.09	15.725±2.45	12.432±2.72
24	1.410±0.08	0.956±0.14	9.375±0.83	7.585±2.56
48	1.119±0.05	0.690±0.14	8.202±0.47	6.786±1.31
72	1.063±0.12	0.611±0.24	5.888±1.86	5.264±0.62
96	1.083±0.03	0.721±0.15	6.615±0.51	5.065±0.91

[a] Data were obtained from page 60 (Table XI) of the study report.

TABLE 7: Total radioactivity concentration of ^{14}C-RH-573 in rat plasma[a]

Time Point (hr)	µg equivalents/gram (mean±SD)			
	Low dose (Group B)		High dose (Group D)	
	Male (n=3)	Female (n=3)	Male (n=3)	Female (n=3)+
1	2.297±0.45	2.192±0.17	14.010±1.72	11.407±1.64
3	1.430±0.53	1.196±0.07	12.950±0.55	15.736±4.22
6	0.547±0.25	0.514±0.09	7.070±3.13	6.572±2.50
24	0.070±0.01	0.080±0.01	0.876±0.15	0.757±0.17
48	0.046±0.01	0.050±0.01	0.342±0.07	0.367±0.10
72	0.031±0.01	0.031±0.01	0.489±0.33	0.263±0.05
96	0.011±0.00	0.014±0.00	0.115±0.02	0.109±0.02

[a] Data were obtained from page 61 (Table XII) of the study report.

The mean pharmacokinetic parameters for ^{14}C-RH-573 equivalents are summarized in Table 8. T_{max} in blood and plasma was reached at 1 hour post-dose in both sexes of the low-dose group and at 1.7 hours and 3 hours in males and females of the high-dose group, respectively. The

initial elimination half-life ($t_{1/2}$ initial) was rapid, ranging from 3-4 hours in the low-dose group and 5-6 hours in the high-dose group; however, elimination was biphasic and the terminal half-life ($t_{1/2}$) was approximately 29 hours in the low-dose groups and 28-29 hours in the high-dose groups.

Table 8: Mean pharmacokinetic parameters of ^{14}C-RH-573 equivalents in rat blood and plasma[a]

PK Parameter	Unit	Blood				Plasma			
		Low Dose (Group B)		High Dose (Group D)		Low Dose (Group B)		High Dose (Group D)	
		Male (n=3)	Female (n=3)	Male (n=3)	Female (n=3)	Male (n=3)	Female (n=3)	Male (n=3)	Female (n=3)
$t_{1/2}$ initial	hr	NA	NA	NA	NA	3.85	3.24	6.24	5.14
$t_{1/2}$ terminal	hr	NA	NA	NA	NA	28.8	29.1	28.5	27.8
T_{max}	hr	1	1	1.7	3	1	1	1.7	3
C_{max}	µg/g	3.38	3.04	21.2	20.8	2.30	2.19	14.2	15.7
AUC_{0-t}[b]	hr*µg/g	127	83.8	787	665	16.2	15.5	167	158
AUC_{0-inf}[b]	hr*µg/g	NA	NA	NA	NA	16.8	16.1	174	163

NA – Not enough data points were available, terminal elimination was not well defined, to allow a meaningful estimate.
[a] Data were obtained from page 62 (Table XIII) of the study report.
[b] Due to the biphasic nature of the concentration versus time curve, two elimination half-lives were estimated based on each phase of the curve.

B. METABOLITE CHARACTERIZATION STUDIES: A total of twenty-three metabolites were detected in urine and feces samples during the initial HPLC radioprofiling. Retention times obtained during the HPLC radioprofiling were generally similar to those obtained during the LC/MS and LC/MS/MS analyses.

1. **Urine:** The percent distribution of metabolites detected in urine at 0-24 hours post-dosing is summarized for Group A and Group C in Table 9. No parent compound was identified in urine samples during radioprofiling and its absence was further confirmed by LC/MS. Metabolite M1 accounted for 20.86-23.29% of the radioactive dose and metabolite M12 for 9.73-22.67% of the dose. Metabolite M3 accounted for 4.33-5.63% of the dose and M9 for 2.42-6.18% of the dose. Each of the other metabolites accounted for <3% of the dose.

TABLE 9: Profile of metabolites detected in urine of rats dosed with ^{14}C-RH-573[a]

Metabolite ID	Retention Time (min)	Percent of radioactive dose administered (0-24 hours)			
		Group A (low dose)		Group C (high dose)	
		Male (n=4)	Female (n=4)	Male (n=4)	Female (n=4)
M1[b]	2.8-4.2	23.29	20.86	22.66	22.18
M2	4.0-5.0	1.53	2.27	2.04	1.59
M3[c]	4.9-6.5	4.83	4.33	5.63	5.51
M4[c]	7.1-8.9	1.59	2.46	2.34	ND
M5[c]	7.6-8.9	1.95	ND	ND	2.11
M6[c]	8.3-9.7	2.06	1.09	1.01	0.83
M7[d]	11.4-13.3	ND	0.96	0.69	0.76
M8[e]	12.6-14.7	ND	1.20	ND	0.88

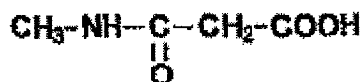
TABLE 9: Profile of metabolites detected in urine of rats dosed with ^{14}C-RH-573[a]

Metabolite ID	Retention Time (min)	Percent of radioactive dose administered (0-24 hours)			
		Group A (low dose)		Group C (high dose)	
		Male (n=4)	Female (n=4)	Male (n=4)	Female (n=4)
M9[f]	14.7-17.6	6.18	4.21	3.62	2.42
M12[c]	23.9-27.0	22.67	16.32	10.20	9.73
Total		**64.10**	**53.70**	**48.19**	**46.01**

ND – Not detected
[a] Data were obtained from page 66 (Table XVII) and pages 72-73 (Table XXIII) of the study report.
[b] Identified as N-methyl-malonamic acid.
[c] A structure has been proposed for the metabolite.
[d] The isolate is composed of two compounds (M7-A and M7-B). A structure has been proposed for both compounds.
[e] The isolate is composed of two compounds (M8-A and M8-B). A structure has been proposed for both compounds.
[f] The isolate is composed of two compounds (M9-A and M9-B). A structure has been proposed for both compounds.

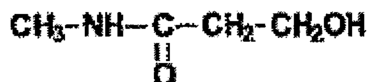
Metabolites M1, M3, M4, M5, M6, M7, M8, M9, and M12 in urine were further analyzed for characterization. M1 was identified as N-methyl-malonamic acid. M7, M8, and M9 were found to consist of two compounds. Structures were proposed for each of these metabolites or their components, as outlined in Appendix A of this report.

The M1 isolate was analyzed by LC/MS using NP-HPLC (LC/MS Condition 2). The molecular weight and HPLC retention time of M1 were consistent with the reference standard for N-methyl-malonamic acid. LC-ESI(+)/MS/MS analysis also yielded product ions similar to those of N-methyl-malonamic acid. Although not distinctive, M1 was also directly detected from the urine sample using LC/MS (Condition 1). Based on these data, M1 was identified as N-methyl-malonamic acid.



M1

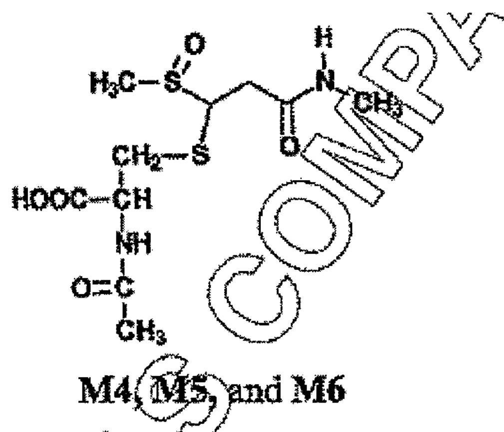
M3 was further analyzed by LC/RAM/(+)ESI-MS (Condition 3). Based on the LC/MS/MS data, M3 was identified as N-methyl-3-hydroxyl-propionamide, although not distinctive, M3 was then detected using LC/MS (Condition 2).



M3

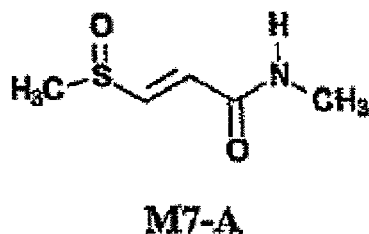
A direct LC/MS (Condition 1) analysis of metabolites M4, M5, and M6 showed distinctive peaks. The results indicated that these compounds were conjugated metabolites. MS/MS

analyses of the compounds yielded similar fragment ions. Therefore, the three metabolites were considered to be conjugated isomers. M4, M5, and M6 were proposed as mercapturic acid conjugates of RH-573 after reductive ring cleavage, followed by S-methylation and oxidation to form sulfoxide. A structure was proposed for these metabolites, although the conjugation position and stereo confirmation could not be assigned from the available data.

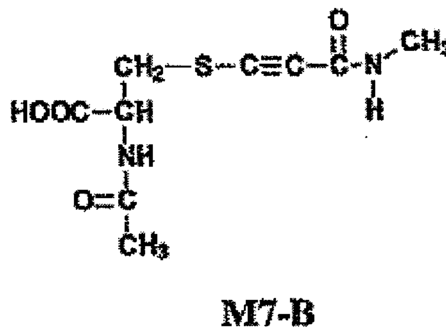


M4, M5, and M6

A direct LC/MS analysis (Condition 1) of M7 showed two distinctive peaks, designated as M7-A and M7-B. Based on these data and MS/MS analyses, M7-A was proposed to be formed from reductive S-N bond cleavage of RH-573, followed by S-methylation and oxidation to form sulfoxide. M7-B was proposed to be formed from an oxidation product (sulfone) of the parent compound.

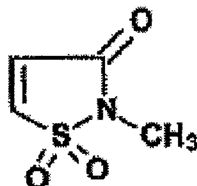


M7-A

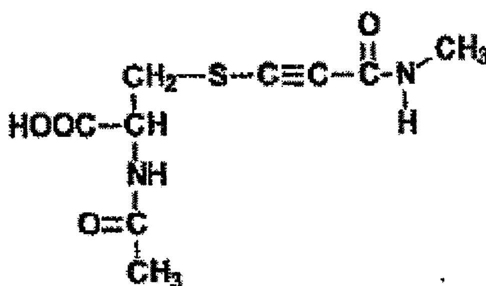


M7-B

A direct LC/MS analysis (Condition 1) of M8 showed two distinctive peaks, designated as M8-A and M8-B. Based on these data and MS/MS analyses, M8-A was proposed to be formed from oxidation of RH-573. M8-B was characterized as a conjugated metabolite of the parent compound.

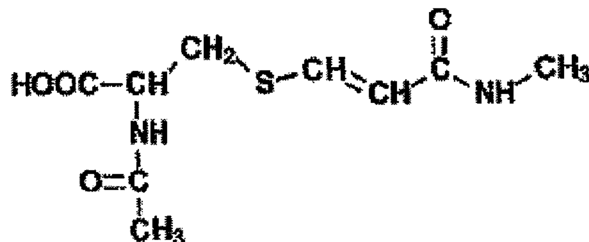


M8-A



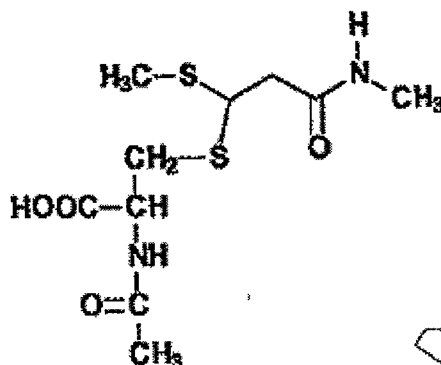
M8-B

NP-HPLC (LC/MS Condition 2) analysis of M9 showed two peaks; the results indicated the two compounds were conjugated metabolites, designated as M9-A and M9-B. Based on MS/MS analyses in the positive ion mode, the two components were considered to be conjugated isomers. APCID-LC/(+)/ESI/MS/MS of a product ion from the two compounds supported the theory they were isomers. M9-A and M9-B were proposed as a pair of mercapturic acid conjugates of RH-573 after the loss of the sulfur atom from the isothiazolinone ring. The conjugation position and stereo confirmation could not be assigned from the limited data. Based on reported findings that glutathione conjugation favors on the β-position of an α, β-unsaturated carbonyl group, the isomer was considered to be a stereo isomer rather than a positional isomer. Analysis of M9-B using LC/MS (Condition 6) supported the proposed structure below:



M9-A and M9-B

M12 was analyzed using LC/ESI(+)-MS/MS (Condition 1), LC/RAM/ESI(+)/MS (Condition 1), and LC/MS Condition 7. Purified M12 was subjected to NMR analysis (performed by Rohm & Haas) for confirmation of its structure.



M12

The urinary metabolites that were identified by name and/or structure comprised approximately 95.8 to 97.6% of the metabolites isolated from urine. The identified metabolites comprised approximately 44.42 to 62.6% of the radioactive dose.

Although metabolite M2 was not characterized based on urinary samples, it was characterized for feces extract samples.

2. **Feces:** The percent distribution of metabolites detected in extracted feces samples is summarized for Group A (low dose) and Group C (high dose) in Tables 10 and 11, respectively. The metabolites are identified by retention time. No parent compound was identified in the feces. The results of a fortified feces extraction sample indicated that ^{14}C-RH-573 remained stable during the extraction procedure. Metabolite M2 accounted for 12.20-25.78% of the radioactive dose. Each of the other metabolites accounted for less than 2% of the dose.

TABLE 10: Profile of metabolites detected in feces of rats in Group A (low dose) dosed with ^{14}C-RH 573[a]

Metabolite ID	Retention Time (min)	Percent of radioactive dose administered					
		Male (n=4)			Female (n=4)		
		0-24 hr	24-48 hr	Total 0-48 hr	0-24 hr	24-48 hr	Total 0-48 hr
M2[b]	4.0-5.0	11.71	0.49	12.20	11.12	2.21	13.33
M3	4.9-6.5	ND	ND	ND	0.28	ND	0.28
M10	~17.5	ND	ND	ND	0.23	ND	0.23
M12	23.9-27.0	ND	0.06	0.06	ND	ND	ND
M16	~38.5	ND	ND	ND	ND	0.08	0.08
M17	~39.5	ND	ND	ND	0.25	ND	0.25
M18	~40	0.36	ND	0.36	0.23	0.09	0.32
M19	~41.5-42.5	0.30	ND	0.30	ND	0.08	0.08
M20	~43	ND	ND	ND	0.23	0.09	0.32
M21	~44	ND	ND	ND	0.30	0.15	0.45
Total		**12.37**	**0.55**	**12.92**	**12.64**	**2.70**	**15.34**

ND – Not detected
[a] Data were obtained from page 71 (Table XXII) and pages 72-73 (Table XXIII) of the study report with the exception of the 0-48 hr totals, which were calculated by the reviewer.
[b] The isolate is composed of four compounds (M2-A, M2-B, M2-C, and M2-D). Structures have been proposed for M2-B and M2-C.

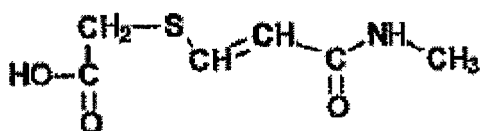
TABLE 11: Profile of metabolites detected in feces of rats in Group C (high dose) dosed with ^{14}C-RH 573[a]

Metabolite ID	Retention Time (min)	Percent of radioactive dose administered					
		Male (n=4)			Female (n=4)		
		0-24 hr	24-48 hr	Total 0-48 hr	0-24 hr	24-48 hr	Total 0-48 hr
M2[b]	4.0-5.0	21.29	0.78	22.07	24.17	1.61	25.78
M3	4.9-6.5	0.53	0.06	0.59	1.26	0.20	1.46
M11	~20.5	ND	0.07	0.07	ND	ND	ND
M12	23.9-27.0	ND	0.07	0.07	ND	ND	ND
M13	~31	ND	ND	ND	0.48	ND	0.48
M14	~33.5	ND	ND	ND	0.61	0.04	0.65
M15	~37.0	ND	ND	ND	ND	0.05	0.05
M16	~38.5	0.45	ND	0.45	ND	ND	ND
M17	~39.5	0.65	ND	0.65	ND	ND	ND
M19	~41.5-42.5	ND	ND	ND	ND	0.04	0.04
M22	~44.5	ND	ND	ND	ND	0.06	0.06
M23	~45.5	0.53	ND	0.53	ND	0.05	0.05
Total		**23.45**	**0.98**	**24.43**	**26.52**	**2.05**	**28.57**

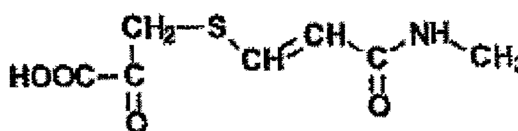
ND – Not detected
[a] Data were obtained from pages 71 (Table XXII) and pages 72-73 (Table XXIII) of the study report with the exception of the 0-48 hr totals, which were calculated by the reviewer.
[b] The isolate is composed of four compounds (M2-A, M2-B, M2-C, and M2-D). Structures have been proposed for M2-B and M2-C.

Metabolite M2 was further analyzed for characterization. M2 was isolated using preparative

HPLC (HPLC Condition 3). Analysis by NP-HPLC under acidic conditions (LC/MS Condition 2) found M2 to contain one minor and three major components. LC/MS analysis (Condition 4) yielded four components, designated as M2-A, M2-B, M2-C, and M2-D. Based on the data, structures were proposed for M2-A and M2-C. The parent compound can undergo reductive ring cleavage, followed by glutathione conjugation, which can be further metabolized to form a cysteine conjugate. S-substituted cysteines may undergo transamination to form corresponding thiopyruvic acids, which are then metabolized to thiolactic and thioacetic (thioglycolic) acids. The conjugation position and stereo confirmation could not be determined from the available data. The position is most likely to be on C_5 rather than C_4 according to published findings that glutathione conjugation favors the β-position of an α, β-unsaturated carbonyl group Structures could not be determined for M2-B or M2-D



M2-B



M2-C

Although metabolites M3 and M12 were not characterized based on feces extracts, structures were proposed for these fractions based on urine samples.

The M2 metabolite characterized for feces comprised 86.9 to 94.4% of the metabolites isolated from feces.

3. <u>Metabolic Pathways</u>:

The main metabolic pathways of RH-573 in the rat were proposed as follows:

- Phase I metabolites derived from:
  - oxidative cleavage of the isothiazolinone moiety with the loss of the sulfur atom and the C_4, C_5 double bond reduction of the moiety (M1 and M3)
  - oxidation of the intact parent compound (M8A)
  - reductive cleavage of the S-N bond, followed by methylation and oxidation (M7A)
- Phase II metabolites derived from:
  - reductive cleavage of the S-N bond, followed by methylation and oxidation (M7A), followed by glutathione conjugation, with further degradation of the glutathione moiety to yield a cysteine conjugate, which then formed a mercapturic acid moiety (M4, M5, and M6)
  - reductive cleavage of the S-N bond, followed by methylation and glutathione conjugation, with further metabolism of the glutathione moiety to yield a cysteine conjugate, which then formed a mercapturic acid moiety (M12)
  - reductive cleavage of the S-N bond, followed by the formation of mercapturic acid conjugate (M9)
  - oxidation of the intact parent compound, followed by loss of sulfur

dioxide, then formation of mercapturic acid conjugate (M7B and M8B)
- reductive cleavage of the S-N bond, followed by the formation of cysteine conjugate, then transamination of the cysteine moiety (M2C), followed by decarboxylation and further oxidation (M2B)

III. DISCUSSION AND CONCLUSIONS:

A. CONCLUSIONS: The investigators conclude that RH-573 was rapidly excreted from the rat; greater than 80% of the administered dose was eliminated within 24 hours post-administration. A majority of the radioactivity was recovered in urine and cage rinse; a lesser amount was recovered in the feces.

In the low-dose group, T_{max} in blood and plasma was reached for both sexes at one hour post-dosing. In the high-dose group, T_{max} was reached at 1.7 hours in males and at 3 hours in females. The elimination half-lives of ^{14}C-label from plasma ($T_{1/2}$ initial) were rapid and ranged from 3 to 6 hours.

RH-573 was extensively metabolized and excreted mainly into the urine following a single dose. Intact RH-573 was not found in the urine or feces.

N-methyl-malonamic acid (M1) and a 3-mercapturic acid conjugate of 3-thiomethyl-N-methyl-propanamide (M12) were the major components in urine samples, accounting for 20.9%-23.3% and 9.7%-22.7% of the dose, respectively.

The initial HPLC radiochromatography indicated the presence of at least 23 components derived from RH-573. All metabolites accounting for >1% of the administered dose were identified and/or characterized by LC/MS and LC/MS/MS. M12 (3-thiomethyl-N-methyl-propanamide) was also confirmed by 1D and 2D-NMR.

The metabolites of RH-573 are comprised of a variety of Phase I metabolites consisting of reductive and oxidative cleavage products of RH-573 and Phase II metabolites consisting of mercapturic acid conjugates of Phase I metabolites. The formation of mercapturic conjugates from RH-573 was supported by the findings of many glutathione conjugates and its related conjugates in rat bile from a bile-cannulated rat metabolism study of RH-573.[1]

There were no significant sex differences in the metabolic profiles. There was a slight difference between dose groups: higher amounts of 3-mercapturic acid conjugate of N-methyl-2-propenamide (M9) and 3-mercapturic acid conjugate of 3-thomethyl-N-methyl-propanamide (M12) conjugated metabolites were detected in Group A (low-dose) rats.

B. STUDY DEFICIENCIES: The following deficiencies are based on the requirements for a Tier I study under OPPTS 870.7485.

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[1] MRID 47154010. Wu, D. and H. Kim-Kang (in progress) Metabolism of ^{14}C-RH-573 in the biliary cannulated rat. XBL Study No. 04043, Report No. RPT01215.

Major Deficiencies:

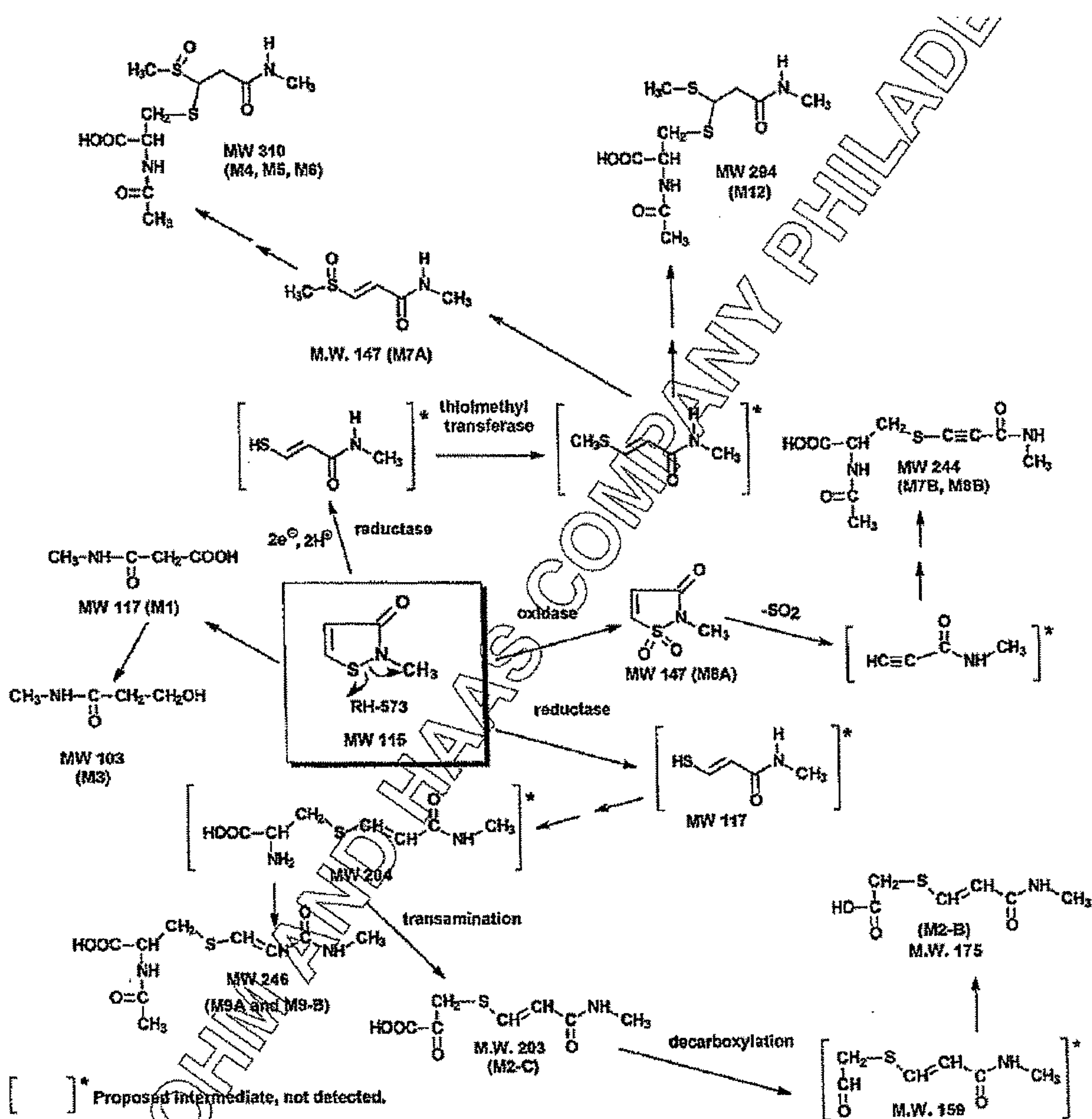
- Radioactivity in urine was not determined at 6 and 12 hours on day 1 of collection.
- Expired air was not collected. No explanation was provided for not measuring radioactivity in expired air; 92-96% was recovered in urine, cage wash, and feces.

Minor Deficiencies:

- The rationale for the selection of the doses was not fully explained.
- The method of assignment of animals to test groups was not explained.

D. STUDY CLASSIFICATION: This metabolism study in the rat is classified **ACCEPTABLE - NONGUIDELINE** and does not satisfy the guideline requirement for a metabolism study [OPPTS 870.7485, OECD 417] in the rat. The study is upgradable if justification is provided for not collecting expired air samples or urine samples at 6 and 12 hours post-dosing and/or if these requirements are waived. **The absence of these data does not significantly affect the results of the study.**

Appendix A
Proposed Metabolic Pathway for RH-573



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