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DATA EVALUATION RECORD

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

PC CODE: 107104 **DP BARCODE:** 341456

TEST MATERIAL (PURITY): [^{14}C] 2-methyl-isothiazolin-3-one (~99% a.i.)

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

CITATION: Kim-Kang, H., and D. Wu (2005). Metabolism of ^{14}C-RH-573 in the biliary cannulated rat. XenoBiotic Laboratories, Inc., Plainsboro, NJ 08536. XBL Report Number: RPT01215, July 14, 2005. MRID 471540-10. Unpublished.

SPONSOR: Rohm and Haas Company, Spring House, PA 19477-0904

EXECUTIVE SUMMARY: In a metabolism study of [^{14}C]-RH-573 in the biliary cannulated female rat (MRID 471540-10), ^{14}C-RH-573 (99% pure) was administered by gavage in a single dose of 50 mg/kg. The excretion and metabolite profiles were investigated in bile, urine, and feces. Initially, some 31 metabolites were found and characterized.

The test material was rapidly excreted following a single gavage administration, and was eliminated primarily (about 49%) through the urine. Lesser amounts were excreted (about 29% and 6%) via biliary and fecal routes respectively. Primary excreted compounds in the urine following a single oral exposure included the metabolized parent compound (M-1) as N-methyl malonamic acid and (M-12) as the 3-mercapturic acid conjugate of 3-thiomethyl-N-methyl-propionamide.

The metabolites of RH-573 were a large number of a variety of Phase I metabolites. These consisted of reductive and oxidative cleavage products as well as Phase II products consisting of glutathione and/or glutathione-derived conjugates of Phase I metabolites of RH-573. Glutathione and glutathione conjugates, and di-conjugates with glucuronic acid were also found in the bile samples.

The study was able to provide proposed metabolic pathways for the metabolism of RH-573 in the female rat.

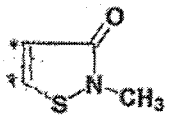
This metabolism study is considered to be **ACCEPTABLE - GUIDELINE** only for metabolite profiling because it satisfies the guideline requirement for the metabolic profiling portion of a metabolism study OPPTS 870.7485.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality statements are provided.

I. MATERIALS AND METHODS

A. MATERIALS:

1. **Test compound:**

Radiolabelled test material:	[4,5-^{14}C] – 2-methyl-isothiazolin-3-one
Radiochemical purity:	96.90% as determined by HPLC (stock solutions were on average 95.8%)
Specific activity:	48.50 mCi/g
Lot/batch #:	1063.0004 - a wet white solid
Structure:	 * ^{14}C position
Non-Radiolabelled test material:	RH-573
Description:	A clear liquid, colorless to brown. Stored at room temperature, with a vapor pressure of 4 x 10^{-6} mmHg at 20°C and a melting point of 56.3°C.
Lot/batch #:	8001J123 (TD# 01-119)
Purity:	~51.4%
Contaminants:	Not Reported
CAS # of TGAI:	Not Reported

2. **Vehicle and/or positive control:** NANOPure® water was the vehicles used in the study.

3. **Test animals:**

Species:	Rat
Strain:	Sprague-Dawley - females with bile ducts cannulated
Age/weight at study initiation:	females: about 10 weeks of age the body weights ranged from 251-276 g.
Source:	Hilltop Lab Animals, Inc. (Scottdale, PA)
Housing:	Rats were housed in polycarbonate cages (1 per cage) and changed to Nalgene metabolism cages during the testing
Diet:	Rats were provided Certified [Purina Rodent Chow # 5002 diet] *ad libitum*; the chemical analysis of the feed indicated that there were no contaminants that would effects the outcome of the study.
Water:	Tap water was provided *ad libitum;* from a bottle dispenser on the cage during acclimation. Concentrations of chemical

	contaminants provided in the analyses of water samples were below detection limits. Electrolytes were supplemented during the study.
Environmental conditions:	**Temperature:** 19-25°C **Humidity:** 40.0-70.0% **Air changes:** 10-15/hr **Photoperiod:** 12 hrs dark/12 hrs light
Acclimation period:	Animals were acclimatized ≥2 days to laboratory and 1 day to the metabolism cages

4. **Preparation of dosing solutions:** The stock solution was made with: 0.18556 grams of RH-573 with a potency of 51.4% and about 240 µCi of carbon labeled RH-573 being added to a dosing bottle. NANOPure® water was added and mixed to dissolve the chemical to achieve a concentration of 10 mg/mL. The volume administered amounted to ~5.0 mL/kg of rat body weight based on dose levels of 50 mg base-eq/kg.

   Concentration of the dosing solution was assayed both pre- and post-dosing by weighing duplicate aliquots of 50 µL of the dosing solution into a 5 mL volumetric flask and bringing to volume with water. Following mixing, duplicate samples of 100 µL were analyzed in a liquid scintillation spectrometer.

   Stability of the test substance in solution was tested and confirmed by the laboratory using HPLC. Fractions were collected from the HPLC output every 30 seconds into minivials. Each vial representing a fraction was then read for radioactivity by Liquid Scintillation Counting.

B. STUDY DESIGN AND METHODS:

1. **Group arrangements:** Levels of radioactivity were determined in urine, feces, bile, and cage washings for each of the 4 individual female rats. Please see Table 1.

TABLE 1: Dosing groups of metabolic characterization studies for RH-573 in a biliary cannulation study in Rats.			
Test group	**Dose of labeled material (mg base-eq/kg)**	**Number/sex**	**Remarks**
Oral dose	50	4 females	Animals were sacrificed 24 hr post-dose

2. **Dosing and sample collection:** For Metabolite Characterization Studies

Dosing: The study employed a single oral gavage dose of the test material. The test material was loaded into a syringe and weighed before being introduced into the mouth of the test animal. Following deposition of the test material, the syringe was again weighed to determine the exact amount of volume given.

Sample collections: The bile, feces, and urine were collected as pre-dose and post-dose samples. Bile was collected from 0.5 hr before to the 0 start time as a pre-dose sample. Pre-dose collections of urine, feces, and cage washings were carried out for 24 hours before the given dose.

During the study, the samples were collected into tarred containers and freeze-trapped using dry ice to limit atmospheric oxidation, bacterial degradation and evaporation. The cage washing at the end of the study was made with isopropanol and water (1:1). The washings samples were collected in pre-weighed containers.

Sample preparation for Radioactivity Analysis: Duplicate sample aliquots of bile (each ~ 0.025 mL), urine (each ~ 0.1 mL), and cage washing (each ~ 1.0 mL) were weighed into scintillation vials, and assayed with scintillation cocktail directly by liquid scintillation counting (LSC). Feces were homogenized with 3 to 5 x (w/v) NANO Pure® water and produced triplicate aliquots of homogenate (each about equal to 100 mg of fresh feces weight) which were combusted in a Harvey Biological Oxidizer and then counted by (LSC).

Radioactivity Analysis: Levels of radioactivity were counted directly for the bile, cage rinse, urine, and feces extract samples in a liquid scintillation counter (LSC). Levels of activity in the feces extracts were found by combusting aliquots of the homogenized samples in the Harvey OX-500 or the OX-300 Biological Sample Oxidizer and counting the ^{14}C-labeled CO_2 in 15 mL of Harvey Scintillation Carbon-14 Cocktail. Combustion analysis samples were made in triplicate and direct counting was in duplicates. Product identification and profiling of metabolites was limited to single or duplicate samples depending on sample availability. Liquid scintillation counting was generally completed on a Beckman liquid scintillation counter for 10 minutes or until the 2-sigma error was less than or equal to 2% (whichever came first). Fractions collected from HPLC were determined by utilizing 2 minutes of counting.

Urine, bile, cage rinse, and feces were counted for total radioactive residue levels by setting the scintillation spectrometer to zero background. Both pre-dose and treated samples were

counted for each matrix and the counts per minute (cpm) were converted to disintegrations per minute (dpm). The dpm from the pre-dose sample was subtracted from the treated sample counts and the net dpm per aliquot was used for further calculations.

Metabolite Profiling: For the profiling purposes, individual urine and proportionally pooled samples of the collected urines were directly analyzed by liquid scintillation counting. Feces collected over the 24 hour period were homogenized and proportionally pooled. A sample of the pooled homogenate was extracted with methanol by shaking and then centrifuging. Following several repetitions of an extraction and centrifugation process the supernatant was then counted by LSC. Triplicate aliquots of the dried post extraction solids (PES) were combusted and assayed by LSC.

Effluent fractions were collected for 30 seconds each and the bile and feces extracts were examined by LSC.

Urinary metabolite radio-profiles of ^{14}C-RH573 were produced on a Waters® 2695 HPLC system which was coupled to a Packard Radiomatic Series 500TR Flow Scintillation Analyzer online using the HPLC condition 2

The radio-profiles of metabolites of ^{14}C-RH-573 in feces were produced on a Waters 2695 HPLC system using the HPLC condition 2. The samples were 30 second fractions which were examined by LSC. HPLC radio-chromatograms were constructed using Microsoft® Excel software. The radioactivity peaks were integrated to determine the percent distribution of individual radioactive peaks or regions in each sample.

In addition to determining the peaks and percentages of radioactivity in the individual fractions collected, the process of identification of the individual metabolites was attempted. The method utilized was that of that of using liquid chromatography coupled with mass spectrometry. The LC/MS methodology was termed Condition 1 and is seen below.

Since metabolites isolated from the bile samples were found to be unstable, both urine and bile samples were analyzed directly by LC/MS.

3. **Statistics:** The calculations that were employed in the study were made for the most part using an electronic calculator or an IBM-compatible PC with Microsoft® Excel software. Calculations were standard computations for parameters such as the dpms, calculations of the dosing suspension concentrations, etc., and included means and standard deviations which are common calculations to this type of study.

II. RESULTS:

1. **Excretion** A summary of the percent of the doses that were recovered from the collections of urine, bile, and feces that were pooled, as well as the amount of dose that had adhered to the cage as a rinse can be seen in the Table 2. The pooled samples were radio-profiled using RP-HPLC-Condition 2 (reversed phase HPLC).

Table 2 – Summary Percent of Dose Recovered from Urine, Cage Rinse, Bile and Feces[a]					
Animal ID Group A	**% in Urine**	**% in Feces**	**% in Cage Rinse**	**% in Bile**	**Total % Recovered**
GA-001-F	47.90	3.92	6.05	27.47	85.34
GA-002-F	50.74	3.52	1.88	29.88	86.02
GA-003-F	51.43	5.71	3.00	32.08	92.22
GA-004-F	46.90	11.41	3.80	26.94	89.05
Mean±SD	**49.24±2.18**	**6.14±3.64**	**3.68±1.76**	**29.09±1.76**	**88.16±3.15**

[a] Data obtained from page 44 of the report.

The data that was produced on the 0-24 hour feces extraction sample can be found below in Table 3. It shows that only 6.14% of the gavage dose was passed through the intestinal tract in the 24 hour period following dosing. It also shows that 2.23% did not extract from the feces as noted by the PES Fraction.

Table 3 – Extraction Summary of 0-24 hr Feces Sample [a]							
Group / Sex	**Interval**	**AVG. % Dose[1]**	**Extract Fr. (%)[2]**	**PES (%)[3]**	**% Recovery[4]**	**% Dose in Extract Fr.[5]**	**% Dose in PES Fr.[6]**
Group A / Females	0-24	6.14%	63.65%	36.35%	96.65%	3.91%	2.23%

[a] Data obtained from page 48 of the report.
[1] Average % dose in the pooled feces sample; refer to Table 1
[2] Fractional distribution of the radioactivity in the extract
[3] Fractional distribution of the radioactivity in PES fraction
[4] Extraction recovery calculated using the TRR value
[5] Dose in the extract; analyzed by HPLC for profiling
[6] Dose in the PES fraction; was not further analyzed
NA - Not Applicable

B. METABOLITE CHARACTERIZATION STUDIES:

1. **Distribution of metabolites:** The distribution of the 31 metabolites that were found in the bile, urine, or feces as a percentage of the original dose can be seen in Table 4 below.

Table 4 – Percent Distribution of 14C-RH-573 Metabolites in Urine, Bile, and Feces Expressed as % Dose [a]				
Metabolite	**Retention Region R_t(min)[1]**	**Urine**	**Feces**	**Bile**
		GA-F-U24	**GA-F-F24[2]**	**GA-F-B24**
		%Dose	**%Dose**	**%Dose**
M1	2.8-4.2	23.11	ND	ND
M2	4.0-5.0	2.50	3.21	0.82
M3	4.9-6.5	5.86	0.52	ND
M4	7.0-8.9	2.31	0.17	ND
M5	7.6-9.2	0.57	ND	ND
M6	8.3-9.8	0.79	ND	ND
M7	11.4-13.3	0.53	ND	ND
M8	12.6-14.7	0.78	ND	ND
M9	14.7-17.6	2.44	ND	ND
M10	17.5-18.2	0.37	ND	ND
M11	20.5-21.5	0.42	ND	ND
M12	23.9-27.0	9.54	ND	ND
M13	30-30.5	ND	ND	0.64
M14	32.5-34.5	ND	ND	0.76
M15	~37.0	ND	ND	0.50
M24+M24B[1]	~5	ND	ND	3.30
M25A+M25B[1]	~7	ND	ND	3.02
M26	~8.5	ND	ND	1.25
M27A+M27B[1]	~9.5	ND	ND	0.74
M28A+M28B[1]	~10.5	ND	ND	2.83
M29	~12.5	ND	ND	0.44
M30A+M30B[1]	~13.5	ND	ND	0.84
M31	~16.5	ND	ND	4.87
M32	~19	ND	ND	2.33
M33	~20	ND	ND	1.81
M34	~23.5	ND	ND	2.05
M35	~25	ND	ND	0.65
M36	~26.5	ND	ND	0.87
M37	~28.5	ND	ND	0.41
M38	31.0-32.0	ND	ND	0.61
M39	35.0-36.0	ND	ND	0.35
TOTAL:		**49.24**	**3.9%**	**29.09%**

[a] Data obtained from page 47 of the report
Note: ND = Not Detectable
% Dose value may vary ± 0.02% due to rounding
[1] The radioactive peak was shown to contain two compounds by LC/MS analysis.
% Dose
[2] Refer to Table 2 for % dose in feces extract.
Note: M7, M8, and M9 were shown to be composed of at least two compounds.

The different metabolites were identified by LC/MS and are shown in the tables on pp 49, 50, and 51 of the study report.

Further evaluation of the metabolites when the molecular weights were much greater than the parent compound warranted the further use of additional MS investigations. These instances then used the LC/MS/MS systems to further investigate the possible structure of the metabolite/s present. This occurred often with the suggestions of occurrences of conjugation and isomers.
For the structures see the proposed metabolic pathways found on pp 16 of the report and extracted for inclusion below.

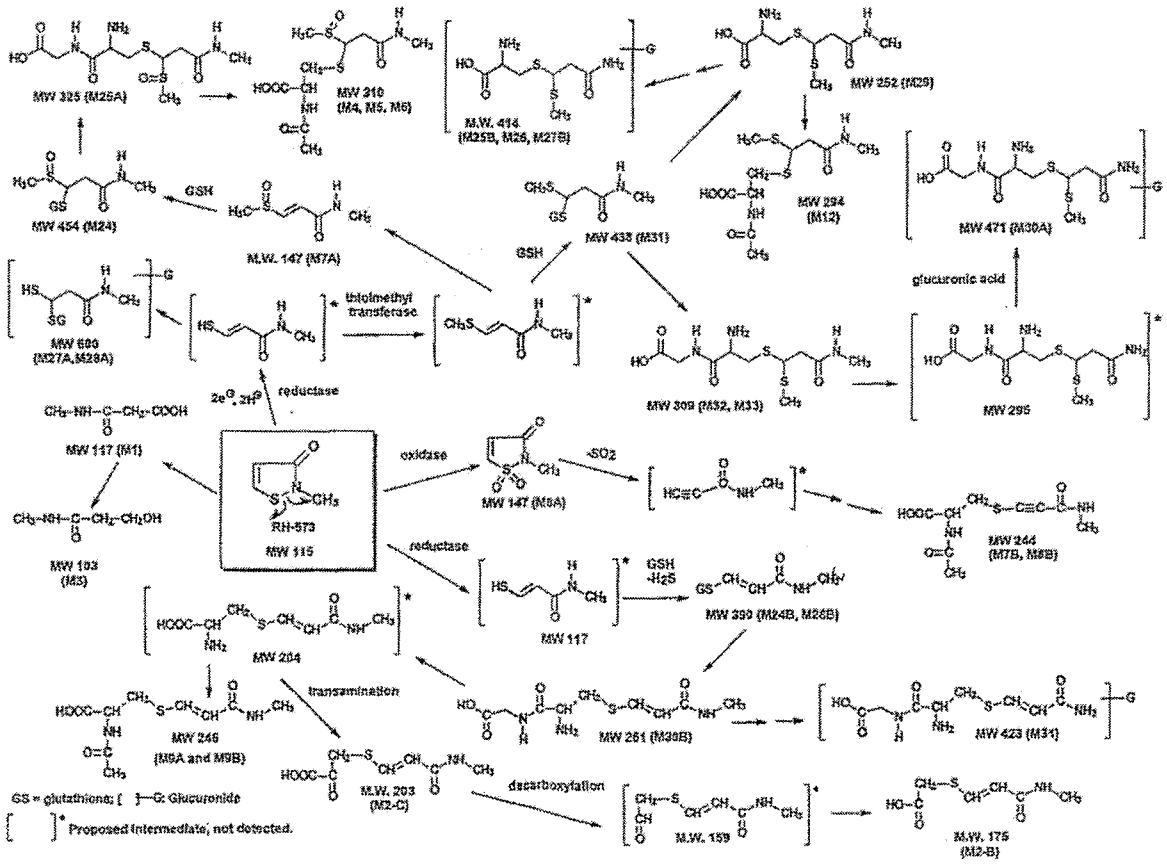
Table 5. Metabolite

Metabolite	Condition	Rationale	Found in
M-1	N-Methyl malonamic acid	Found in another rat metabolism study – confirmed (MRID 47154009[1])	urine
M-2A,B,C,D	Major component	Found in another rat metabolism study –well studied (MRID 47154009)	feces
M-3	-	Found and studied in another rat metabolism study (MRID 47154009)	urine
M-4,5,6	Conjugates	MS/MS –proposed as mercapturic acid conjugates of RH-573	urine
M-7A	sulfoxide	Added two oxygens	urine
M-7B	conjugate	Molecular weight increased 129 above parent	urine
M-8A	Oxidation of RH-573	Molecular wt. increased 32 above parent	urine
M-8B	conjugate	Molecular wt 129 higher than parent	urine
M-9	Conjugate(mercapturic)	Molecular wt 131 higher than parent	urine
M-12	Conjugate with addition of sulfinyl or S-methyl	Molecular wt 180 higher than parent and 48 higher than M-9. Has been studied thoroughly in other metabolism study (MRID 47154009)	urine
M-24	Conjugate with glutathione	Molecular wt is 339 higher than the parent and 307 higher than M-7A indicating conjugation of glutathione to M7A	bile
M-24B M-24B	M-24 loss of glutamyl moiety	Co-eluted - Molecular wt was 64 less than that of M-24 showing a loss of the glutamyl moiety	bile
M-25A	M-24 loss of glutamyl moiety	Co-eluted with M-25B, molecular wt was 129 lower than M-24 showing a loss of the glutamyl moiety	bile
M-25B	Proposed to be glucuronic aid conjugate	Co-eluted with M-25A Molecular wt was 299 higher than parent. Suggesting a glucuronic acid conjugate	bile
M-26	Proposed to be a glucuronic acid conjugate	Considered to be a glucuronic acid conjugate	bile

[1] 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.

M-27A	Co-eluted with M-27B	Same as M-27B	bile
M-27B	Co-eluted with M-27A	Molecular wt was 299 higher than parent Considered to be a glucuronic acid conjugate	bile
M-28A	Co-eluted with M-28B	Molecular wt was 485 higher than RH-573 indicating that they were conjugates. Both M-27A and M-28A were considered to be glucuronic acid conjugates on different positions	bile
M-29	Proposed to be a conjugated metabolite	Molecular wt was 137 higher than RH-573 indicating a conjugate	bile
M-30A	Proposed to be a conjugated metabolite	Molecular wt was 356 higher than RH-573 indicating a conjugate, but MS/MS suggested the loss of glucoronide moiety	bile
M-30B	Proposed to be a conjugated metabolite	Molecular wt was 146 higher than RH-573 but MS/MS suggested a loss of water and a loss of NH_2CH_3	bile
M--31	Proposed to be a conjugated metabolite	Molecular wt was 323 higher than RH-573 but MS/MS indicated a loss of water and NH_2CH_3	bile
M-32 M-33	Proposed to be conjugated metabolites And are stereoisomers	Molecular wt was 194 higher than RH-573, MS/MS produced fragment showing loss of water and loss of $HSCH_3$	bile
M-34	Proposed to be a conjugated metabolite	Molecular wt was 308 higher than RH-573 indicating it to be conjugated metabolite. Possibly a conjugate of glucuronic acid	bile

Proposed Metabolic Pathways of RH-573 in Biliary Cannulated Rats



III. DISCUSSION AND CONCLUSIONS:

A. CONCLUSIONS:

- The study author concluded that the test material was rapidly excreted from the rat. Twenty four hours after dosing s greater than 88% of the dose was eliminated. A majority of the dose was excreted via the urine with lesser amounts in the bile, feces and cage rinse.

- RH-573 was extensively metabolized and excreted mainly in the urine following a single oral dose to the rat. Intact RH-573 was not found in urine, bile, or feces.

- Metabolite M-1, N-methyl malonamic acid and M-12, 3-mercatureic acid conjugate of 3-thiomethyl-N-methyul-propionamide were the major components of the 0-24 hour urine sample and accounted for about 23.1 and 9.5% of the dose, respectively. The 24 hour bile sample contained a large number of compounds, each account for less than 5% of the dose. Only the glutathione conjugate of 3-thiomethyl-N-methyl-propionamide (M-31) accounted for 4.9% of the dose.

- The initial HPLC radio-chromatography revealed the presence of at least 31 components derived from RH-573. All the metabolites accounting for greater than 1% of the administered dose were identified and/or characterized by LC/MS and LC/MS/MS. Some of the minor metabolites accounting for less than 1% of the dose were also identified and/or characterized by LC/MS and LC/MS/MS.

- 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 glutathione or glutathione-derived conjugates of Phase I metabolites of RH-573. In addition to glutathione conjugates, di-conjugates with glucuronic were also found in bile samples. The proposed metabolic pathways of RH-573 in rats are presented in Figure 44 in the report.

- The finding of glutathione and its related conjugates in bile during the study supported the finding of mercapturic acid conjugates as major metabolites in urine during the previous rat metabolism study (MRID 47154009).

  Since the non-cannulated rat passes the bile back to the intestinal tract which has the effect of microbial metabolism, these further metabolized products can in a large number of instances be reabsorbed back into the animal body. They may well be metabolites to which the body is ultimately exposed and not just the "31" metabolites that were found in this study. As a result, the metabolism could also include the exhalation of ^{14}C-carbon dioxide as well as the 3 monitored excretion routes,

C. STUDY DEFICIENCIES: The following study deficiencies are noted:

- The study did not evaluate the non-cannulated female rat, or the male to see if any differences between the sexes did occur in metabolism of the RH-573; however, MRID 47154009 data illustrates that little sexual differences in metabolism of RH-573 are observed.
- Rationale for the dose of unlabelled RH-573 was not provided.
- The mineral supplementation of the drinking water was not provided in detail or the rationale for it.

D. <u>STUDY CLASSIFICATION</u>: This study in the rat is **ACCEPTABLE - GUIDELINE** and satisfies only the guideline requirement for the metabolite characterization portion of a metabolism study (870.7485). This study is only a single part of a two-part requirement to evaluate the metabolism of the chemical under (870.7485).