

STUDY PLAN

(Copy No. 1/1)

STUDY TITLE

**VALIDATION OF ANALYTICAL METHOD FOR THE DETERMINATION OF
CAPTAN METABOLITE-THPI-1,2,3,6-TETRAHYDROPHthalimide,
CAPTAN METABOLITE-3-OH THPI AND CAPTAN METABOLITE-5-OH THPI
IN BLOOD BY GC-MS/MS**

Study No.: AG-G1809**SPONSOR**

**SHARDA CROPCHEM LIMITED
PRIME BUSINESS PARK
DASHRATHLAL JOSHI ROAD
VILE PARLE (WEST)
MUMBAI - 400056
INDIA**

TEST FACILITY

**EUROFINS ADVINUS AGROSCIENCES SERVICES INDIA PRIVATE LIMITED
PLOT Nos. 21 & 22, PHASE II, PEENYA INDUSTRIAL AREA
DISTRICT-BENGALURU URBAN,
BENGALURU-560058
KARNATAKA
(INDIA)**

TABLE OF CONTENTS

UNITS AND ABBREVIATIONS	3
1. BASIC STUDY INFORMATION	4
2. OBJECTIVE.....	4
3. TEST SYSTEM AND ITS JUSTIFICATION.....	4
4. TEST GUIDELINE.....	5
5. GOOD LABORATORY PRACTICES	5
6. MATERIALS AND METHODS	5
6.1 Captan metabolite-THPI-1,2,3,6-Tetrahydrophthalimide Reference Standard Details	5
6.2 Captan metabolite-3-OH THPI Reference Standard Details.....	6
6.3 Captan metabolite-5-OH THPI Reference Standard Details.....	6
6.4 Apparatus/Chemicals	7
6.5 Residue analysis in samples	7
6.6 Preparation of Solutions for analysis	7
6.7 Data Presentation.....	8
7. QUALITY ASSURANCE	8
8. AMENDMENT PROCEDURES	8
9. SAFETY PRECAUTIONS	8
10. REPORTING	8
11. ARCHIVING	9
12. STUDY PLAN DISTRIBUTION	9
13. REPORT DISTRIBUTION.....	9
14. DISPOSAL OF SAMPLES.....	9
15. REFERENCES.....	9
16. AGREEMENT	10
ANNEXURE 1. ANALYTICAL METHOD VALIDATION	11

UNITS AND ABBREVIATIONS

°C	: Degree celcius
µL	: Microliter
mL	: milliliter
mg	: milligram
ng	: nanogram
Conc.	: Concentration
RSD	: Relative Standard Deviation
RPM	: Revolution per Minute
Vol.	: Volume
mg/L	: milligram/liter
QN/QL	: Quantitative/Confirmative
OECD	: Organization for Economic Co-operation and Development
GC-MS/MS	: Gas Chromatography with tandem mass spectrometry
LOD	: Limit of Detection
LOQ	: Limit of Quantification
R	: Replication
STD.	: Standard

1. BASIC STUDY INFORMATION

Monitoring Scientist & Sponsor : 5.1.2.e
Representative Sharda Cropchem Limited, Prime
Business Park,
Dashrathlal Joshi Road
Vile Parle (West), Mumbai – 400056,
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Telephone: 5.1.2.e
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Study Director :

5.1.1.c

Study Code by Test Facility : RESMV

Study Schedule (dd/mm/yyyy)

Study Initiation Date : 30/08/2023

Experimental Start Date : 04/09/2023

Experimental Completion Date : Latest by 30/10/2023

First Draft Report : Latest by 30/11/2023

Final Report : Latest by 30/12/2023 or the date on
(Study Completion Date) which the study director signs the final
report, but no later than 180 days from
the issue of the first draft report.

2. OBJECTIVE

The objective of the study is to validate the analytical method for determining the Captan metabolite-THPI-1,2,3,6-Tetrahydrophthalimide (THPI), Captan metabolite-3-OH THPI (3-OH-THPI) and Captan metabolite-5-OH THPI (5-OH-THPI) in blood in accordance with the guidance document SANTE/2020/12830, Rev.2 of the European Commission and Primary methods. The intended limit of quantification (LOQ) for THPI, 3-OH-THPI and 5-OH-THPI is 0.01 mg/L.

3. TEST SYSTEM AND ITS JUSTIFICATION

Animal blood will be considered as test system as per study requirement.

Whole blood sample of animal origin will be sourced from Adgyl Lifesciences Private Limited (located in same premises) and assumed to have no residues of Captan metabolite-THPI-1,2,3,6-Tetrahydrophthalimide (THPI), Captan

metabolite-3-OH THPI (3-OH-THPI) and Captan metabolite-5-OH THPI (5-OH-THPI) and used for blank and fortified controls. Source details will be included in the raw data and presented in the final report.

4. TEST GUIDELINE

The study will be conducted in accordance with the following guideline:

SANTE/2020/12830, Rev.2 (14. February 2023): Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes.

5. GOOD LABORATORY PRACTICES

The study will be performed in accordance with the following:

- OECD Principles of Good Laboratory Practice (as revised 1997), ENV/MC/CHEM(98)17, OECD, Paris, 1998, which are compatible with the U.S. EPA FIFRA (40 CFR Part 160) Good Laboratory Practice Standards.
- EC Council Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonization of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (codified version), Official Journal of the European Union L50, 20.2.2004 P. 44-59.

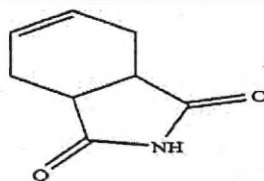
This study will also be conducted as per the mutually agreed study plan and the test facility's standard operating procedures.

6. MATERIALS AND METHODS

The materials that will be used for the study are the general laboratory equipment/instruments and chemicals and glassware and the details of which will be recorded in raw data and given in the report of the study.

6.1 Captan metabolite-THPI-1,2,3,6-Tetrahydrophthalimide Reference Standard Details

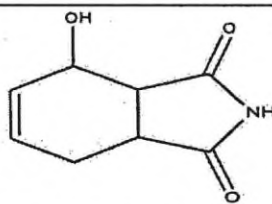
The reference standard details are given below as per the certificate of analysis:

Name	:	Captan metabolite-THPI-1,2,3,6-Tetrahydrophthalimide
Chemical Name	:	3a,4,7,7a-tetrahydro-1H-isondole-1,3(2H)-dione
Appearance	:	Off-White Solid
Structure	:	
Source	:	Varanous Labs Private Limited

CAS No.	:	1469-48-3
Batch No.	:	VL-0813
Formula	:	C ₈ H ₉ NO ₂
Molecular Weight	:	151.16 g/mol
Purity by HPLC	:	99.80
Retest Date	:	17/04/2025
Storage Temperature	:	For long term storage 2-8°C

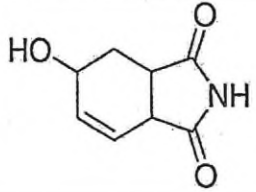
6.2 Captan metabolite-3-OH THPI Reference Standard Details

The reference standard details are given below as per the certificate of analysis:

Name	:	Captan metabolite-3-OH THPI
Chemical Name	:	(4RS)-4-hydroxy-3a,4,7,7a-tetrahydro-1H-isoindole-1,3(2H)-dione
Appearance	:	White Solid
Structure	:	
Source	:	Varanous Labs Private Limited
CAS No.	:	886463-87-2
Batch No.	:	VL-0811-F
Formula	:	C ₈ H ₉ NO ₃
Molecular Weight	:	167.16 g/mol
Purity by HPLC	:	99.93%
Retest Date	:	17/04/2025
Storage Temperature	:	For long term storage 2-8°C

6.3 Captan metabolite-5-OH THPI Reference Standard Details

The reference standard details are given below as per the certificate of analysis:

Name	:	Captan metabolite-5-OH THPI
Chemical Name	:	(5RS)-5-hydroxy-3a,4,5,7a-tetrahydro-1H-isoindole-1,3(2H)-dione
Appearance	:	White Solid
Structure	:	

Source	:	Varanous Labs Private Limited
CAS No.	:	1935025-01-6
Batch No.	:	VL-0812-F
Formula	:	C ₈ H ₉ NO ₃
Molecular Weight	:	167.16 g/mol
Purity by HPLC	:	99.73%
Retest Date	:	17/04/2025
Storage Temperature	:	For long term storage 2-8°C

6.4 Apparatus/Chemicals

The following contains a partial list of apparatus / general chemical used in this study. Any apparatus/chemical used in this study and not contained in the following list can be found in the appropriate sections of this report.

- Balances
- General glassware
- GC column
- Gas Chromatograph with a Mass Spectrometer
- Micropipettes
- Centrifuged
- Ultrasonic Bath
- Milli-Q® water
- Anhydrous magnesium sulphate
- Sodium chloride
- N, O-bis(trimethyl silyl) trifluoroacetamide (BSTFA)
- Trimethylchlorosilane (TMCS)
- Acetonitrile

6.5 Residue analysis in samples

Methods will be validated for Captan metabolite-THPI-1,2,3,6-Tetrahydrophthalimide (THPI), Captan metabolite-3-OH THPI (3-OH-THPI) and Captan metabolite-5-OH THPI (5-OH-THPI) as per SANTE/2020/12830, Rev.2 for the quantification of residues in blood. The details of method validation parameters are given in Annexure 1.

6.6 Preparation of Solutions for analysis

Reference Standard Stock

A required quantity of reference standards Captan metabolite-THPI-1,2,3,6-Tetrahydrophthalimide (THPI), Captan metabolite-3-OH THPI (3-OH-THPI) and Captan metabolite-5-OH THPI (5-OH-THPI) will be weighed into separate volumetric flasks of known volume and diluted to the volume with suitable solvent.

Samples will be treated with 70 µL N, O-bis(trimethyl silyl) trifluoroacetamide (BSTFA) containing 10% trimethylchlorosilane (TMCS) to form the volatile trimethylsilyl derivatives of captan metabolites. Samples will be derivatized by

heating for 30 minutes at $115^{\circ}\text{C} \pm 2^{\circ}\text{C}$. Finally, the samples will be analysed by GC-MS/MS.

Note: If any other procedure is followed for the preparation of stock solution, it will be recorded in study records and reported.

6.7 Data Presentation

The results will be compiled, summarised and presented in the report.

7. QUALITY ASSURANCE

The quality assurance unit of Eurofins Advinus Agroservices India Private Limited, Bengaluru will inspect critical phases of the study; review the raw data, the draft and final report. These inspections and audits will be carried out by quality assurance personnel, independent of the staff involved in the study. The findings of all inspections will be reported to the management and to the study director. Inspection dates and reporting dates will be presented in the report as a quality assurance statement signed by the responsible QAU. The quality assurance unit has reviewed the study plan and will receive a copy thereof.

8. AMENDMENT PROCEDURES

This study plan may be amended by the study director. All amendments will describe the change(s), the reason(s) for the amendment, and the effect on the study, if any.

If immediate action is necessary, e-mail-based agreement received from the monitoring scientist with approval authority will be considered and it will be confirmed as soon as possible by study plan amendment. The amendment will be distributed and added to copies of the study plan.

Any unintended change(s) to the study plan will be documented in the raw data and mentioned in the report as deviation(s).

9. SAFETY PRECAUTIONS

Gloves, goggles and face mask (if required), in addition to protective body garments and shoes will be used to assure enough personnel health and safety. In case of skin contact, wash immediately with plenty of water and soap. In case of eye contact, flush eyes immediately with plenty of water and provide immediate medical aid.

10. REPORTING

The final report will include, but not be limited to:

- The name and address of the sponsor and the test facility.
- The study schedules.
- The list of scientists / professionals.
- The objectives stated in the study plan.
- A description of all methods with references, wherever applicable.
- A description, summary and assessment of all results.

- All deviations from study plan, if any.
- CoA of reference standard used for the study.
- A summary of all study plan amendments, if any.
- A description of all circumstances that may have affected the quality or integrity of the study.
- The quality assurance statement signed by the quality assurance unit.
- The GLP compliance statement signed by the study director.
- GLP Certificate.

Note: The final report will be issued no later than 180 days from the issue of first draft report to the sponsor. If a response from sponsor for finalization of draft report is not received within this stipulated time, management will approve the finalization of report and archiving. In such an event, any subsequent requests from sponsor for modification, correction or addition to the final report will be subject to report amendment at additional cost.

11. ARCHIVING

After the completion of the study, the following will be archived for 9 years at the contracted archiving facility, Adgyl Lifesciences Private Limited: raw data, study plan, study plan amendment (if any), draft and final reports.

12. STUDY PLAN DISTRIBUTION

The signed study plan will be distributed as follows:

- a) Test facility: Signed study plan in original – Copy No. 1/1.
- b) Sponsor: The signed study plan as a PDF will be provided to sponsor electronically.

13. REPORT DISTRIBUTION

The signed final report will be distributed as follows:

- a) Test facility: One signed final report in original – Copy No. 1/1.
- b) Sponsor: Upon study completion, the signed final report as a PDF will be provided to sponsor electronically.

14. DISPOSAL OF SAMPLES

After experimental completion, all the samples generated during the conduct of the study will be disposed suitably and the same will be documented in the raw data.

15. REFERENCES

1. OECD 1998, OECD Principles of Good Laboratory Practice, In: OECD Environmental Health and Safety Publications, Series on Principles of Good Laboratory Practice and Compliance Monitoring, No. 1 ENV/MC/CHEM (98)17, Paris 1998.
2. US-EPA 1989, Environmental Protection Agency, 40, CFR Part 160 FIFRA, Good Laboratory Practice Standard, Final Rule.

16. AGREEMENT

This study plan for Study No. AG-G1809 has been mutually agreed and the final study plan was approved by the Monitoring Scientist & Sponsor Representative through e-mail on 22/08/2023 (dd/mm/yyyy).

5.1.2.e
[Redacted]
5.1.2.e
[Redacted]

STUDY DIRECTOR

DATE: 30/08/2023
(mm/dd/yyyy)

5.1.2.e
[Redacted]
5.1.2.e
[Redacted]

Date: 01/09/2023
(DD/MM/YYYY)

5.1.2.e
[Redacted]
5.1.1.c
[Redacted]

Date: 01/09/2023
(DD/MM/YYYY)

ANNEXURE 1. ANALYTICAL METHOD VALIDATION

1. INTRODUCTION

The analytical method for the determination of Captan metabolite-THPI-1,2,3,6-Tetrahydrophthalimide, Captan metabolite-3-OH THPI and Captan metabolite-5-OH THPI residues in blood will be validated according to SANTE/2020/12830, Rev.2 by establishing specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), matrix effect, accuracy (as % recovery) and precision (as % rsd).

2. SPECIFICITY/SELECTIVITY

The specificity of the method will be determined by analyzing diluent, control matrix samples and standard solution. Analyte identity will be confirmed by retention time match with an analytical standard solution.

Interference (if any) should not exceed 30% of the total peak area measured for the target analyte at the limit of quantification (LOQ) level.

3. CONFIRMATION

Two mass transitions will be evaluated and respective chromatograms along with product ion mass spectrum will be shown in the report. One of the two mass transitions will be proposed as quantification transition but both selected mass transitions should be applicable interchangeably for quantification and confirmation.

4. MATRIX EFFECT

Matrix effects on detection will be evaluated by preparation of matrix-matched standard solutions and comparing the peak area or slope in matrix to peak area or slope of solvent based standard solutions at linearity range of concentrations. Matrix-matched standards should be prepared using the same percentage (at least 90% of matrix load) in the final volume of a control sample extract.

$$\text{Matrix effects (\%)} = 100 \times \text{peak area or slope (matrix)} / \text{peak area or slope (solvent)} - 100$$

If overall matrix effects are greater or equal to 20%, matrix-matched standard solutions must be used for quantification. Otherwise, either solvent-based or matrix-matched standard solutions may be used.

5. LINEARITY OF DETECTOR RESPONSE AND RANGE

The linearity of the detection system will be demonstrated by use of a standard curve covering a working range of not more than 30% of the LOQ to 20% above the highest level in sample extract. A minimum of five working standard solutions will be prepared from a stock solution of Captan metabolite-THPI-1,2,3,6-Tetrahydrophthalimide, Captan metabolite-3-OH THPI and Captan metabolite-3-OH THPI analysed in triplicate by GC-MS/MS respectively under the given conditions. The peak area of each injection will be recorded. A linear graph will be plotted between concentration and peak area response of standard solutions to get a calibration curve. Intercept (a), slope (b) and correlation coefficient (r) values will also be calculated. From the calibration curve, the concentration range for which

the detector response is linear will be selected. The r^2 or r value generated from the calibration curve data should be ≥ 0.98 or 0.99 . If considered necessary, $1/x$ or $1/x^2$ weighting may be applied.

6. LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTIFICATION (LOQ)

The limit of detection (LOD) will be estimated to one-third of LOQ or lowest calibration standard in matrix. The limit of quantification (LOQ) 0.01 mg/L will be established based on the fortification level at which analyte concentration at which an acceptable accuracy and precision will be obtained.

The actual LOD and LOQ sample preparation will be captured in the raw data and presented in the report.

7. ACCURACY (% RECOVERY)

An aliquot of blood in five replications will be fortified separately at LOQ (0.01 mg/L) level. The fortified blood samples along with two control samples will be extracted and analyzed as per details given in Section No. 10. The method will be considered acceptable if the obtained per cent recoveries are in the range of 70 to 120%.

8. PRECISION (%RSD)

From the "Mean $\pm$ s.d." of the accuracy test, the precision of the method as % rsd will be calculated.

$$\text{Precision (as \% rsd)} = \frac{s.d.}{\text{Mean}} \times 100$$

The method will be acceptable if % rsd $< 20\%$.

9. EXTRACT AND STANDARD STABILITY

Final Extract Stability

To prevent degradation, final extracts will be stored in a freezer at $< -18^\circ\text{C}$. If final extracts are not analyzed within 24 hours, the stability of the analyte is sufficiently proven, if the recoveries in the fortified samples are within the acceptable range of 70-120%, measured against freshly prepared standards.

Sample extracts fortified at the $10 \times \text{LOQ}$ level will be stored in a deep freezer at $< -18^\circ\text{C}$ along with one control sample. After the storage period, the samples will be reanalyzed by single injection to confirm their stability.

Standard Stability

To prevent degradation, standard stock solutions (stock, calibration etc.) will be stored in a fridge or freezer. The stability of an existing standard will be checked by preparing a new stock standard and comparing the detector responses. The means from at least 5 replicate measurements for each of the two solutions should not differ by more than 10%. Stability of standard stock solutions need not to be demonstrated if standard stock solutions are prepared freshly and analyzed immediately.

10. EXTRACTION PROCEDURE

Captan metabolite-THPI-1,2,3,6-Tetrahydrophthalimide, Captan metabolite-3-OH THPI and Captan metabolite-5-OH THPI will be extracted following the extraction procedure.

An aliquot of 1 mL blood sample will be weighed into a 50 mL centrifuge tube extracted with 10 mL of acidified acetonitrile (1% acetic acid in acetonitrile, v/v), 4 g of anhydrous magnesium sulphate and 1 g of sodium chloride will be added and shaken vigorously for by hand for one minute. The sample will be centrifuged at 4000 rpm for 10 minutes for phase separation and extractant will be filtered through a membrane filter.

The extractant will be evaporated and reconstituted in acetonitrile for derivatization. The acetonitrile solution will be treated with 70 µL N, O-bis(trimethyl silyl) trifluoroacetamide (BSTFA) containing 10% trimethylchlorosilane (TMCS) to form the volatile trimethylsilyl derivatives of captan metabolites. Samples will be derivatized by heating for 30 minutes at 115°C ± 2°C. Finally, the samples will be analysed by GC-MS/MS.

Note: Any change in the extraction procedure will be recorded and reported.

11. CHROMATOGRAPHIC ANALYSIS

The samples will be subjected to GC-MS/MS analysis operated under the following conditions:

Instrument	: Gas Chromatography Equipped with Mass Spectrometer (Triple quadrupole 8050 system Shimadzu) or equivalent
Column	: DB-5 MS, 30m × 0.250 × 1.0 µm or equivalent
Gradient Temperature	: 80°C for 1 min 20°C/min to 180 °C hold for 0 min 40°C/min to 300 °C hold for 4 min
Mode	: Splitless
Injection Volume	: 1 µL
Run Time	: 14 minutes
Carrier Gas	: Helium (He)
Collision Gas	: Argon (Ar)
Ionization mode	: Positive
Ion Source	: EI
Source Temperature	: 280°C

Acquisition Mode		
Compound Name	Ions Monitored <i>m/z</i>	CE
Captan metabolite-THPI-1,2,3,6-Tetrahydrophthalimide	208 - Quantification	20.0
	207 - Confirmation	20.0
Captan metabolite-3-OH THPI	296 - Quantification	20.0
	142 - Confirmation	20.0
Captan metabolite-5-OH THPI	168 - Quantification	20.0
	106 - Confirmation	20.0

Note: Any change in the conditions and ion transitions will be recorded and reported.

12. CALCULATIONS

A linear graph will be plotted between concentration and peak area response of standard solutions to get a calibration curve. Intercept (a), slope (b) and correlation coefficient(r) values will be calculated.

The concentration (graph conc.) of Captan metabolite-THPI-1,2,3,6-Tetrahydrophthalimide, Captan metabolite-3-OH THPI and Captan metabolite-5-OH THPI in the fortified samples will be calculated by substituting the observed peak area response (Y) of each sample solution in the linear equation, $Y = a + bX$ (developed from the calibration curve), where Y denotes peak area response and X denotes the concentration (mg/L).

Percent recoveries from fortified samples will be calculated following the equation below.

$$\text{Graph Conc. (X)} = \frac{\text{Peak area (Y)} - \text{Intercept (a)}}{\text{Slope (b)}}$$

$$\text{Amount Recovered (mg/L)} = \frac{\text{Graph Conc. (}\mu\text{g/mL)} \times \text{Vol. made up (mL)} \times \text{Vol. of Std. Injected (}\mu\text{L)} \times \text{Vol. of Extract (mL)}}{\text{Vol. of Sample Injected (}\mu\text{L)} \times \text{Aliquot Taken for Dilution (mL)} \times \text{Vol. of Blood (mL)}}$$

$$\text{Amount Fortified (mg/L)} = \frac{\text{Conc. Treatment Solution (mg/mL)} \times \text{Vol. of Fortified Solution (mL)}}{\text{Vol. of Blood (mL)}}$$

$$\text{Recovery (\%)} = \frac{\text{Amount Recovered (mg/L)} \times 100}{\text{Amount Fortified (mg/L)}}$$

Note: The exact calculations if other than above will be recorded and reported.