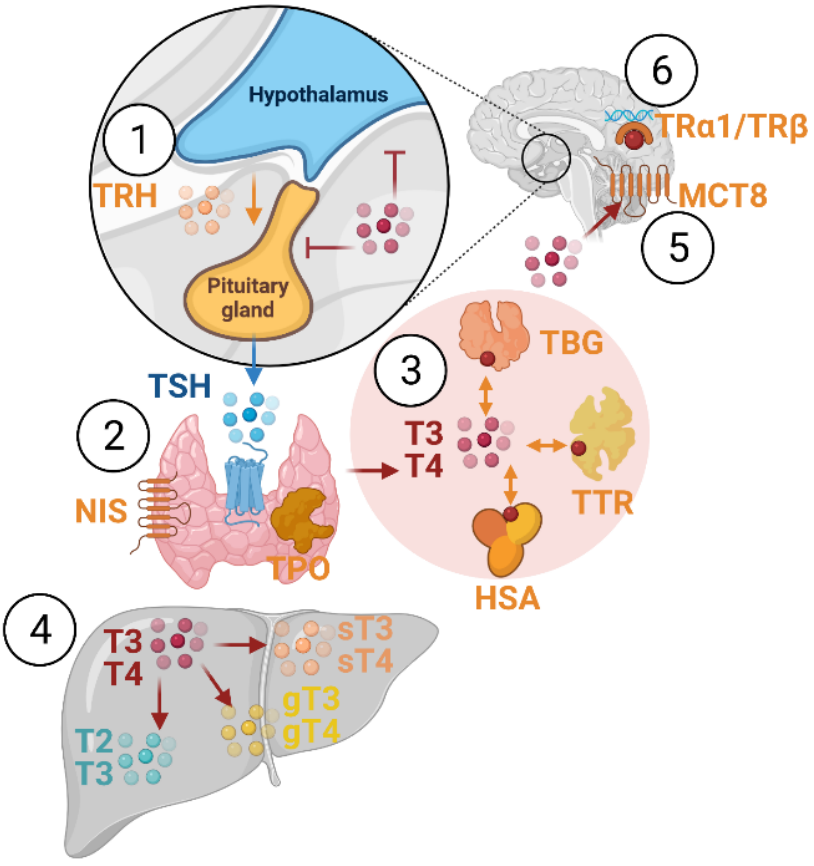
STUDY REPORT

Inhibition of thyroid hormones (THs) glucuronidation using liquid chromatography/mass spectrometry (LC/MS-MS) – Part 1

EURL ECVAM validation study of a battery of mechanistic methods relevant for the detection of chemicals that can disrupt the thyroid hormone system



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This study report has been prepared within the context of a collaboration agreement signed in 2019 with the Joint Research Centre (JRC) Directorate for Health, Consumers and Reference Materials (Chemicals Safety and Alternative Methods Unit F3 / EURL ECVAM), for the validation of mechanistic methods to identify potential modulators of thyroid hormone signalling. For information on the methodology and quality underlying the data presented in this report, users should contact the referenced source.

The study report describes the experimental design and includes data generated in Part 1 of the validation study. The method was developed and experimentally assessed by EU-NETVAL laboratory Accelera, Italy.

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Inhibition of thyroid hormones (THs) glucuronidation using liquid chromatography/mass spectrometry (LC/MS-MS)

Report

Method Number	Study	Status
4b	Part 1	Final

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Signed		
Date	15 February 2023	15 February 2023

Table of Contents Page

SUMMARY

This study was performed as Part 1 of Method 4b, part of the EURL ECVAM coordinated Thyroid Validation Project. The study confirmed the robustness and the reliability of method 4b based on the related standard operating procedure (SOP No. 4b, [9]). T3 and T4, incubated at three different concentrations (2, 5 and 10 µM) with human liver microsomes and cofactor UDPGA, produced a reproducible concentration of glucuronides conjugates in five valid runs (CV% ≤ 20%). The UGTs activity was inhibited by mefenamic acid (test item), but not by fluconazole (negative control). Inhibition levels were reproducible as well and confirmed to be proper controls for Part 2 of the process.

Summary of Experimental Conditions Part 1			
Item	**Description**	**Incubation Conc.**	**Check**
Test system	Human Liver Microsomes	1 mg/mL	UGT activity checked by MU fluorescence assay
Substrates	T3 and T4	2, 5,10 µM	Concentration check of SS by LC-MS/MS
Cofactor	UDPGA	5 mM	-
Test Item	Mefenamic Acid	500, 320, 200,160, 80, 50, 20, 5 µM	Concentration check of WS by LC-MS/MS
Negative Control	Fluconazole	500 µM	-

Summary of Analytical Run of MU	
Linearity	R-squared range 0.09988 from to 0.9998
LLOQ	1.00 µM
ULOQ	80.00 µM
Selectivity	No peak
LQC, MQC, HQC	Inter-run Precision (CV %) = 5.7%, 5.7 %, 3.3% Inter-run accuracy (bias %) = 12.5%, -8.9%, -1.9%

Summary of Analytical Run of T3G and T4G	
Linearity	T3G R-squared range 0.09988 from to 0.9998 T3G Inter-run Precision (CV%) ranged from 7.3 and 12.9% T3G Inter-run Precision (CV%) ranged from -4.2 and 5.3%
	T4G R-squared range 0.09919 from to 0.9973 T4G Inter-run Precision (CV%) ranged from 4.6 and 10.9% T4G Inter-run Precision (CV%) ranged from -8.1 and 6.7%
LLOQ	0.005 µM
ULOQ	2.000 µM
Selectivity	No peak
Carry-over	< LLOQ
LQC, MQC, HQC	Inter-run Precision T3G (CV%) = 11.4%, 10.4 % 9.1% Inter-run accuracy T3G (bias%) = 8.3%%, 5.5%, -0.3%
	Inter-run Precision T4G (CV%) = 8.7%, 8.4 % , 10.7% Inter-run accuracy T4G (bias%) = 12.6%, 7.4%, -5.4%

Summary Data of MU Fluorescent Assay: UGTs activity of HLM characterization					
UGT Activity (nmole/min/mg)					
inter-run		**TI 500 µM**	**NC 500 µM**	**No Solvent**	**Solvent**
New HLM Batch (2 valid runs)	Mean	29.5	132.9	122.8	114.2
	SD	2.9	8.6	1.5	0.4
	CV %	9.8	6.5	1.2	0.3
In parallel of Inhibition Assay (5 valid runs)	Mean	30.4	109.4	110.2	111.3
	CV %	5.3	11.4	6.8	7.9
	Bias%	17.4	10.4	-10.3	-2.6
% Inhibition vs Solvent					
All valid runs	Mean	72.2	0.7	2.1	-

Summary data of inhibition assay on the T3 and T4 glucuronides formation:												
Inter-run	**T3G Concentration (µM)**						**T4G Concentration (µM)**					
	T3 2 µM		**T3 5 µM**		**T3 10 µM**		**T4 2 µM**		**T4 5 µM**		**T4 10 µM**	
	Avg	**CV%**	**Avg**	**CV%**	**Avg**	**CV%**	**Avg**	**CV%**	**Avg**	**CV%**	**Avg**	**CV%**
NO UDPGA	LLOQ		LLOQ		LLOQ		LLOQ		LLOQ		LLOQ	
TI 500 µM	LLOQ		0.007	12.1	0.027	13.1	0.022	18.2	0.065	11.8	0.143	13.4
TI 320 µM	LLOQ		0.021	14.7	0.055	18.9	0.046	16.8	0.118	18.8	0.242	11.6
TI 200 µM	0.009	16.4	0.037	14.0	0.083	16.2	0.069	18.4	0.206	16.0	0.433	16.5
TI 160 µM	0.016	18.2	0.051	9.5	0.101	13.7	0.086	16.8	0.266	17.4	0.549	17.2
TI 80 µM	0.024	17.3	0.074	5.0	0.147	5.7	0.123	14.3	0.381	11.7	0.788	17.8
TI 50 µM	0.026	14.2	0.081	16.8	0.146	10.7	0.124	8.3	0.430	18.0	0.850	14.8
TI 20 µM	0.038	13.7	0.118	13.8	0.187	10.4	0.189	10.9	0.580	12.4	1.202	14.3
TI 5 µM	0.045	7.4	0.130	10.5	0.216	15.3	0.224	9.2	0.685	13.7	1.321	18.6
NC 500 µM	0.046	8.1	0.133	7.6	0.208	13.3	0.233	13.5	0.693	17.2	1.309	16.4
No Solvent	0.050	19.0	0.139	16.9	0.191	14.5	0.204	11.9	0.648	18.4	1.222	15.4
Solvent	0.053	13.5	0.152	13.1	0.201	13.1	0.208	11.1	0.639	18.5	1.200	9.2
Inter-run	**T3G % activity vs solvent**						**T4G % activity vs solvent**					
	T3 2 µM		**T3 5 µM**		**T3 10 µM**		**T4 2 µM**		**T4 5 µM**		**T4 10 µM**	
	Avg	**CV%**	**Avg**	**CV%**	**Avg**	**CV%**	**Avg**	**CV%**	**Avg**	**CV%**	**Avg**	**CV%**
NO UDPGA	LLOQ		LLOQ		LLOQ		LLOQ		LLOQ		LLOQ	
TI 500 µM	LLOQ		5.0	19.1	12.4	19.3	11.2	13.6	10.2	12.7	11.6	15.3
TI 320 µM	LLOQ		13.9	12.9	27.3	15.5	22.1	14.5	18.6	19.9	19.8	18.7
TI 200 µM	18.0	19.1	25.7	6.6	42.4	5.3	34.1	13.5	32.3	5.9	34.8	13.2
TI 160 µM	31.4	18.7	35.5	10.3	52.0	11.4	42.5	6.9	41.6	9.0	43.9	9.4
TI 80 µM	47.3	10.6	52.1	15.3	75.5	7.8	61.5	13.2	59.9	6.0	63.6	8.6
TI 50 µM	48.6	10.6	56.2	6.7	75.0	7.4	62.3	7.8	67.1	4.7	68.3	11.4
TI 20 µM	76.5	7.0	82.4	11.4	95.7	6.2	94.3	6.1	91.1	6.1	96.6	7.4
TI 5 µM	90.4	11.2	90.2	6.1	110.5	8.6	111.9	9.3	107.5	7.4	107.6	8.3
NC 500 µM	94.7	18.1	93.0	14.6	106.2	2.4	116.3	10.8	108.3	9.5	104.7	7.1
No Solvent	100.4	12.6	95.9	5.5	98.1	11.9	101.6	4.9	100.9	4.4	97.8	5.6
Solvent	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0
IC50	**57.9**	**18.9**	**77.9**	**16.0**	**142.7**	**14.5**	**96.0**	**12.2**	**97.7**	**7.3**	**109.5**	**16.9**

1 STUDY SCOPE

This study was performed as Part 1 of Method 4b, part of the EURL ECVAM coordinated Thyroid Validation Project. After the full description of the method was included in the related standard operating procedure (SOP No. 4b [9]), the robustness and reliability of the method was assessed in this study by performing five valid runs using Mefenamic acid as test item.

2 TEST FACILITY / STUDY PERSONNEL

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3 STANDARD OPERATING PROCEDURES

The study was performed as described in the SOP No. 4b: "Determination of the Potential Inhibition of Thyroid Hormones Glucuronidation in Human Liver Microsomes" (Version No. 15 February 2023). The paragraphs of SOP are indicated, but where the adaptations are applied to the procedure a detailed description is reported in this document.

4 MATERIALS, REAGENT AND EQUIPMENT

4.1 Materials

Test Item, Substrates, Metabolites, Reference Item, Negative control and Internal Standard used in this study are described in the following table.

Substrates		
Chemical name or ID	Triiodothyronine (T3)	Thyroxine (T4)
CAS-number	55-06-1	51-48-9
Lot/batch number	ERM-AC469	BCCB2400
Physical state	Solid	Solid
Supplier	JRC	JRC
Purity	97.1	98.6%
H-statements	H302 (100%), H312 (100%), H332 (100%)	H361 (94.23%), H372 (94.23%)
Expiry date	Jan-22	Feb-22

Metabolites		
Chemical name or ID	Triiodo-thyronine glucuronide (T3G)	Thyroxine glucuronide (T4G)
CAS-number	29919-72-0	21462-56-6
Lot/batch number	9-YSS-13-1	6-EKP-111-3
Physical state	Solid	Solid
Supplier	LGC	LGC
Purity	96%	98%
H-statements	N/A	N/A
Expiry date	May-22	July-22

	Test Item	**Negative Control**
Chemical name or ID	Mefenamic Acid	Fluconazole
CAS-number	61-68-7	86386-73-4
Lot/batch number	MKCH3607	LRAC0255
Physical state	Solid	Solid
Supplier	Sigma	Sigma
Purity	100%	99.96%
H-statements	H302 (100%), H361 (24.19%)	H302 (100%), H315 (79.66%), H319 (72.54%), H335 (18.64%), H360 (70.85%)
Expiry date	Oct-23	Dec-23

	Reference Control	**Internal Standard**
Chemical name or ID	4-Methylumbelliferone	Fexofenadine
CAS-number	90-33-5	153439-40-8
Lot/batch number	BCBW9921	0000094355
Physical state	Solid	Solid
Supplier	Sigma	Sigma
Purity	100%	>99%
H-statements	H315 (100%), H319 (100%), H335 (99.12%)	H302 (25%), H312 (25%), H315 (25%), H317 (50%), H319 (25%), H360 (50%)
Expiry date	Apr-22	Nov-24

4.2 Equipment

The following apparatus was used:

Description	**Model**	**Supplier**
Automated Liquid Handling System	Multiprobe II System	Perkin Elmer
Triple Quadrupole Mass Spec	API2000	Applied Biosystems
Binary pump	1100	Agilent
Column Oven	1100	Agilent
Plates Autosampler	CTC pal	CTC Analytics
Fluorescence Microplate Reader	SpectraMax Gemini XPS	Molecular Devices

Details of the analytical conditions are included in the Analytical Procedure [2,4,6].

4.3 Chemicals and Reagents

Identification	Notes	Type
Water	LC-MS Grade	Reagent
Acetonitrile	HPLC gradient grade	Reagent
Methanol	HPLC gradient grade	Reagent
DMSO	Analytical grade	Reagent
NaOH	1.0 N	Reagent
Acetic Acid	≥ 98%	Reagent
Magnesium Chloride	≥ 98%	Reagent
Alamethicin from Trichoderma viride	≥ 98%	Reagent
Trizma-HCl	Bioperformance Certified	Reagent
Uridine 5′-diphospho-glucuronic acid trisodium salt (UDPGA)	≥ 98%	Co-factor
96-well plate (for incubation)	Flat bottom with lid, polystyrene wells	Material
96-well plate (for fluorescence assay)	Black plate with micro-clear and flat bottom	Material
384-well plates (for analysis)	Cone bottom, polypropylene	Material
1.5 mL, 15 mL und 50 mL centrifugation tubes	Conically shaped, polypropylene	Material
Sterile serological pipettes	In polystyrene	Material

4.4 Solutions

4.4.1 Alamethicin solution

Alamethicin from Trichoderma Viride was dissolved in DMSO at 50 mg/mL, vortexed and sonicated for 5 minutes. Aliquots of 70 µL were stored at -20 °C for 6 months.

4.4.2 Cofactor solution (UDPGA)

Cofactor solution was prepared by dissolving approximately 230 mg (accurately weighed) of UDPGA in an appropriate volume of water to obtain a final concentration of 100.0 mM. The solution was freshly prepared on the day of the experiment and kept at 4 °C; just before the experiment the solution was pre-warmed to 37 °C.

4.4.3 Incubation Medium

The incubation medium was prepared with Trizma HCl solution (100 mM) and Magnesium Chloride Solution (2 mM) diluted in ultrapure water. The media supplements volumes and final concentrations are summarized in the following table:

Compound	Conc.	Volume (mL)	Dilute to (mL)	Final Conc. (µM)	Working Solution ID
Tris-HCl	1000 mM	10.0	100	100 mM	Incubation Medium
$MgCl_2$	1000 mM	0.2		2 mM	

The incubation medium was used to prepare the different working solutions (e.g. mefenamic acid, fluconazole, solvent, no solvent control WS) as described in paragraph 5. Alamethicin was added on the day of the experiment to avoid solubility and stability problems.

4.4.4 Incubation Matrix

The incubation matrix consisted of the biological matrix (HLM 1 mg/mL) and UDPGA (5 mM) diluted in the incubation medium. Volumes and final concentrations are summarized in the following table:

Identity	Compound	Conc.	Volume (mL)	Dilute to (mL)	Final Conc.	Working Solution ID
vial HLM	HLM	20 mg/mL	0.5	10.0	1 mg/mL	Incubation Matrix
UDPGA solution	UDPGA	100 mM	0.5		5 mM	

The incubation matrix was used to prepare the analytical samples (calibration standards, quality controls and selectivity samples).

4.4.5 Internal Standard Solutions

Fexofenadine stock solution (SS ISTD) was prepared by dissolving approximately 5.00 mg (accurately weighed) of the compound in an appropriate volume of DMSO to obtain a final concentration of 1.00 mM. Correction for purity was performed. Working solution of Fexofenadine was prepared by diluting the stock solution with acetonitrile according to the following table:

Identity	Compound	Conc. (µM)	Volume (mL)	Dilute to (mL)	Final Conc. (µM)	Working Solution ID
SS ISTD	Fexofenadine	1000	0.5	50.00	10	ISTD WS-A
ISTD WS- A	Fexofenadine	10	20.00	200.0	1.0	Stop Solvent

Stock and working solutions were stored at nominal +4 °C (stable for 3 months) as described in the analytical validation report [5].

4.4.6 Stop Solution

Stop solution was used to block the glucuronidation reaction. It was prepared by diluting Fexofenadine 1 µM in acetonitrile (stop solvent) with 1% acetic acid in water (50:50, v/v). Stop solution and 1% acetic acid in water were freshly prepared on the day of the experiment and kept at +4 °C until use.

4.4.7 Stop Solution with T3 or T4

Two stop solutions with T3 or T4 (10 µM) were prepared according to the following table:

Identity	Compound	Conc. (µM)	Volume (mL)	Dilute to (mL)	Final Conc. (µM)	Working Solution ID
SS T3	T3	10000	0.01	10.0	10.0	Stop Solution with T3
SS T4	T4	10000	0.01	10.0	10.0	Stop Solution with T4

These solutions were prepared on the day of the analysis and used to prepare the analytical samples (calibration standards, quality controls and selectivity samples).

4.5 Software

The computer software used in this study are summarized in the following table:

System Name	Function
Analyst version 1.4.1	For the capture and analysis of the data. Supplier: SCIEX, Toronto, Canada
Analyst version 1.4.1	To perform regression analysis and calculate analytes concentrations. Supplier: SCIEX, Toronto, Canada
Graph Pad Prism 5.2	To perform IC50 curve calculation. Supplier: GraphPad Software

5 SUBSTRATES, METABOLITES, TEST/ REFERENCE ITEM AND NEGATIVE CONTROL

Substrates, metabolites, test item, reference item and negative control stock solutions and working solutions were prepared in different days and the concentrations were checked and then aliquots were stored.

Information about method analysis or storage conditions are reported in the documents as described in the following tables:

ID	Compound	Solutions	Method Analysis	Storage Conditions
Substrates	T3 and T4	Stock Solution	LC-MS/MS [4]	-20°C for 3 months [5]
Metabolites	T3G and T4G	Stock Solution	LC-MS/MS [2]	-20°C for 3 months[3]
Test Item	Mefenamic Acid	Working Solutions	LC-MS/MS [6]	-20°C for 3 months [7]
Reference Item	Methylumbelliferone	Stock Solution	Fluorimetric analysis	4° C for 3 months
Negative Control	Fluconazole	Working Solutions	LC-MS/MS [6]	-20°C for 3 months [7]

5.1 Substrates Solutions

5.1.1 Stock solution Substrates

Stock solutions of T3 and T4 were prepared by dissolving approximately 5.00 mg (accurately weighed) of the compounds in an appropriate volume of NaOH 1.0 N: DMSO (1:1, v:v) to obtain a final concentration of 10.0 mM. These solutions were then diluted at 5.0 and 2.0 mM using the same solvent.

Substrates Stock Solutions				
SS ID	SS Conc. mM	SS used	Volume SS	Volume DMSO:NaOH
T3 or T4 SS1	10.0	-	-	-
T3 or T4 SS2	5.0	T3 or T4 SS1	300	300
T3 or T4 SS3	2.0	T3 or T4 SS1	120	480

The concentrations of these solutions were checked by LC-MS/MS as described in paragraph 7.1. Aliquots of stock solutions (50 µL) were stored at -20 °C for 3 months [4].

5.1.2 Working solution Substrates

On the day of the experiments, T3 and T4 stock solutions at 2.0, 5.0 and 10 mM were diluted in water (4 mL water + 40 µL Stock solutions), to obtain working solutions (0.02, 0.05 and 0.1 mM) at 10-fold higher concentrations than the intended final substrate concentrations in the incubations (2.0, 5.0 and 10.0 µM).

5.2 Test Item Solutions

The inhibition activities of test items were evaluated in a "Range Finding Assay" using a wide range of concentrations. Once the eight concentrations of the test items were chosen, the "main inhibition assay" were performed. Final data must be performed in five valid runs.

5.2.1 Stock solution Test Item for "Range Finding Assay"

Stock solutions of mefenamic acid were prepared by dissolving approximately 30.00 mg (accurately weighed) of the compounds in an appropriate volume of DMSO to obtain a final concentration of 125 mM. This stock solution was then diluted at 50, 12.5, 5.0, 1.25, 0.5, 0.05, 0.005 mM (stable for 3 months at -20°C [7]) as described in the following table:

Test Item Solutions of "Range Finding Assay				
SS ID	**SS Conc. µM**	**SS used**	**Volume SS**	**Volume DMSO**
TI SS1	125000	-	-	-
TI SS2	50000	TI SS1	200	300
TI SS3	12500	TI SS1	50	450
TI SS4	5000	TI SS1	20	480
TI SS5	1250	TI SS1	10	990
TI SS6	500	TI SS5	200	300
TI SS7	50	TI SS5	20	480
TI SS8	5	TI SS7	50	450

5.2.2 Test Item Working solutions for "Range Finding Assay"

Test Item Working Solutions were obtained by dilution of stock solutions in incubation medium (200-times, 250 µL test item stock solution in 50 mL incubation medium) as described in the table:

Test Item Working Solutions of "Range Finding Assay"				
SS ID	**SS Conc. µM**	**WS ID**	**WS Conc. µM**	**TI Incubation Solution Conc. µM**
TI SS1	125000	TI WS1	625	500
TI SS2	50000	TI WS2	250	200
TI SS3	12500	TI WS3	62.5	50
TI SS4	5000	TI WS4	25	20
TI SS5	1250	TI WS5	6.25	5
TI SS6	500	TI WS6	2.5	2
TI SS7	50	TI WS7	0.25	0.2
TI SS8	5	TI WS8	0.025	0.02

The concentration of test item working solutions was checked by LC-MS/MS as described in paragraph 7.1.1.

Aliquots of test item working solutions were stored -20 °C for 3 months [7]. The day of the experiments eight test item working solutions aliquots (one for each concentration) was thawed and vortexed. Alamethicin solution at 50 mg/mL was added to these solutions in order to obtain 25 µg/mL (4.5 µL) and then vortexed and sonicated for 15 minutes at 40°C.

5.2.3 Stock solution Test Item for the "Main Inhibition Assay"

Different concentrations of Mefenamic Acid were used for the "main inhibition assay". The stock solutions at 80, 40 and 20 mM of mefenamic acid were prepared for the main inhibition assay. Five stock solutions were identical to the ones used in the "range finding assay" and have been renamed as shown in the following table.

Test Item Stock Solutions of "Main Inhibtion Assay"				
SS ID	**SS Conc. µM**	**SS used**	**Volume SS**	**Volume DMSO**
TI SS1	125000	-	-	-
TI SS2	80000	TI SS1	320	180
TI SS3	50000	-	-	-
TI SS4	40000	TI SS1	160	340
TI SS5	20000	TI SS1	80	420
TI SS6	12500	-	-	-
TI SS7	5000	-	-	-
TI SS8	1250	-	-	-

5.2.4 Test Item Working solutions for "Main Inhibition Assay"

Mefenamic acid working solutions were obtained by dilution of the three newly prepared stock solutions in incubation medium (200-times, 250 µL of stock solution in 50 mL of incubation medium). The other solutions were the one prepared for the "range finding assay". The concentration of solutions used in the "main inhibition assay" are described in the table:

Test Item Working Solutions of "Main Inhibtion Assay"				
SS ID	**SS Conc. µM**	**WS ID**	**WS Conc. µM**	**TI Incubation Solution Conc. µM**
TI SS1	125000	TI WS1	625	500
TI SS2	80000*	TI WS2	400	320
TI SS3	50000	TI WS3	250	200
TI SS4	40000*	TI WS4	200	160
TI SS5	20000*	TI WS5	100	80
TI SS6	12500	TI WS6	62.5	50
TI SS7	5000	TI WS6	25.0	20
TI SS8	1250	TI WS8	6.25	5

The new test items working solutions (400, 200 and 100 µM) were checked by LC-MS/MS as described in paragraph 7.1.1.

Aliquots of test item working solutions (9 mL) were stored at -20 °C for 3 months [7].

On the day of the experiments eight test item working solutions aliquots (one for each concentration) were thawed and vortexed. Alamethicin solution at 50 mg/mL was added to these solutions in order to obtain a concentration of 25 µg/mL (4.5 µL) and then vortexed and sonicated for 15 minutes at 40° C.

5.3 Negative Control Solution

5.3.1 Negative Control Stock Solution

Stock solutions of fluconazole was prepared by dissolving approximately 10.00 mg (accurately weighed) of the compound in an appropriate volume of DMSO to obtain a final concentration of 125 mM (stable for 3 months at -20°C [7]).

5.3.2 Negative Control Working solutions

Negative control working solutions was obtained by dilution of stock solution in incubation medium (200-times, 250 µL fluconazole stock solution in 50 mL incubation medium).

Aliquots of negative control working solutions (9 mL) were stored at -20 °C for 3 months [7]. On the day of the experiments one aliquot was thawed and vortexed and 4.5 µL of alamethicin solution at 50 mg/mL was added in order to obtain a concentration of 25 µg/mL. The solution was vortexed and sonicated for 15 minutes at 40°C.

5.4 Solvent and No-solvent Control Solutions

5.4.1 Solvent-Control Working Solution

The solvent-control working solutions was prepared with 0.5% DMSO in incubation medium (250 µL of DMSO in 50 mL incubation medium), the same percentage of solvent as the test item and negative controls working solutions.

Aliquots of solvent-control working solutions (9 mL) were stored at -20 °C. On the day of the experiments one aliquot was thawed and vortexed and 4.5 µL of alamethicin solution at 50 mg/mL was added in order to obtain a concentration of 25 µg/mL. The solution was vortexed and sonicated for 15 minutes at 40°C.

5.4.2 No Solvent-Control Working Solution

Aliquots of No solvent-control working solutions (9 mL of incubation medium) were stored at -20 °C. On the day of the experiments one aliquot was thawed and vortexed and 4.5 µL of alamethicin solution at 50 mg/mL was added in order to obtain a concentration of 25 µg/mL. The solution was vortexed and sonicated for 15 minutes at 40°C.

5.5 Reference Control Solutions

5.5.1 Reference Control Stock Solution

Stock solutions of 4-methylumbelliferone were prepared by dissolving approximately 10.00 mg (accurately weighed) of the compound in an appropriate volume of DMSO to obtain a final concentration of 16 mM (stable for 3 months at 4° C).

5.5.2 Reference Control Working Solution

On the day of the experiments methylumbelliferone working solutions 400 µM was obtained by dilution of the stock solution in incubation medium (250 µL methylumbelliferone stock solution in 10 mL incubation medium).

5.6 Metabolites Solutions

5.6.1 Metabolites Stock Solution

Stock solutions of T3G and T4G were prepared by dissolving approximately 2.00 mg (accurately weighed) of the compounds in an appropriate volume of MeOH:H_2O (1:1, v/v) to obtain a final concentration of 100 µM (stable for 3 months at 4° C [3]).

6 TEST SYSTEM

Commercially available cryopreserved human liver microsomes were used as test system. Microsomes were delivered with a Certificate of Analysis that included metabolic competence. Data are summarized in the following table:

Description	**Code/ Lot Number**	**Supplier**
Human Liver Microsomes, 150 donors, Mixed Gender	X008070/QQY	BioIVT
Metabolic Competence	**Certificate of Analysis**	**Acceptance criteria**
Donors	150 donors	≥ 50 donors
Protein Concentration	24.1 mg/mL	≥ 20 mg/mL
UGT activity*	1617 pmol/min/mg	> 900 pmol/min/mg

* rate of formation of 7-hydroxycoumarin glucuronide

The microsomes thawing procedure is described in detail in paragraph 2.3.4.1 of SOP method 4b [9].

7 METHOD

7.1 Concentration check of test item and substrates

The concentrations of test item and substrates solutions were evaluated by LC-MS/MS. After analysis aliquots of these solutions were stored as described in the stability studies [7, 5]

7.1.1 Test Items working solutions concentration check

The highest concentration of mefenamic acid has been determined during method setup by solubility tests and checked by LC-MS/MS.

Mefenamic acid working solutions were prepared as described in the paragraphs 5.2.2 and 5.2.4 and then the concentrations checked by LC-MS/MS standard.

Two mefenamic Acid reference standard (RS1 and RS2) were prepared by dissolving approximately 2.00 mg (accurately weighed) of the compounds in an appropriate volume of incubation medium to obtain a final concentration of 0.1 µM. These solutions were analysed in triplicate.

The working solutions were diluted at 0.1 µM with incubation medium, except for the 0.025 µM solution, which was analysed as is. All solutions were analysed in triplicate and the concentrations were calculated by comparison with the reference standards.

Details of sample preparation and analytical method are described in the analytical procedure [6].

7.1.2 Substrates stock solutions concentration check

T3 and T4 stock solutions were prepared at 2, 5 and 10 mM as described in paragraph 5.1.1. The concentrations were checked by LC-MS/MS with the analytical conditions described in the analytical procedure [4].

For this analysis, stock solutions were diluted at 2, 5, 10 µM in incubation medium and then the same volume of stop solution was added. The concentrations of solutions were evaluated with a calibration curve of T3 and T4 as follows: aliquots of T3 and T4 stock solutions (10 mM, obtained by different weighing with respect of the stock solution used in the inhibition experiments) were diluted in incubation medium. Eight calibration standard working solutions were prepared as described in the following table:

Sample Name	Final Conc. T3 or T4 (µM)	Solutions			Incubation medium
		Name	Conc. (µM)	Volume (µL)	Volume (µL)
STD 1_WS	12.000	SS	100.000	24	20000
STD 2_WS	6.000	STD 1_WS	12.000	500	500
STD 3_WS	3.000	STD 2_WS	6.000	500	500
STD 4_WS	1.500	STD 3_WS	3.000	500	500
STD 5_WS	0.750	STD 4_WS	1.500	500	500
STD 6_WS	0.375	STD 5_WS	0.750	500	500
STD 7_WS	0.188	STD 6_WS	0.375	500	500
STD 8_WS	0.094	STD 8_WS	0.188	500	500

The calibration standard working solutions were diluted with stop solution in duplicate. T3 and T4 stock solutions at 2.0, 5.0 and 10.0 mM were diluted at 2.0, 5.0 and 10 µM with incubation medium (20 µL of SS in 20 mL of incubation medium). These working solutions were diluted with stop solution in triplicate and the concentrations were calculated by comparison with the calibration standards.

7.2 Characterization of UGTs activity of the test system

The UGTs activity was evaluated previous being used in the inhibition study via 4-methylumbelliferone fluorescence assay. Methylumbelliferone is a UGT substrate and fluorogenic compound that is converted into its non-fluorescent glucuronide conjugate. UGTs specific activity was calculated by comparing the fluorescence loss versus a control performed in the absence of the required cofactor UDPGA. For the evaluation of modulation of UGTs activity, the test item at high concentration and a negative control (Mefenamic Acid and Fluconazole, respectively) were included.

7.2.1 Procedure: Methylumbelliferone Fluorescence Assay of New HLM Batch

The Methylumbelliferone incubations were performed with automated liquid handling system (Multiprobe II System, Perkin Elmer). The determination of UGTs activity of the HLM Batch QQY was determined in two validated runs. The procedure of methylumbelliferone fluorescence assay was described in detail in SOP 4b, paragraph 2.5.4 [9].

The Test item at highest concentration, Solvent-Control, No solvent Control Working solution were thawed, vortexed and an alamethicin solution at 50 mg/mL (see paragraph 5) added in order to obtain a final concentration of 25 µg/mL. Solutions were vortexed, sonicated for 15 minutes at 40°C and then dispensed in the incubation plates.

Microsomes were thawed, diluted to 20 mg/mL as described in paragraph 2.3.4.1 of SOP method 4b [9] and dispensed in the incubation plate (final concentration of 1 mg/mL). A pre-incubation of 15 ± 5 min was performed to aid pore-forming activity of alamethicin.

UDPGA solution was prepared as described in 4.4.2 and dispensed quickly (column by column) in the all wells (5 mM). The addition of the substrates to the first well was documented as the starting incubation time.

These incubations were transferred into a reading plate, diluted 5-fold with the same working solution and incubated with methylumbelliferone (80 µM). This plate was immediately read at the plate reader fluorometer. Fluorescence was measured at Ex/Em = 372/445 nm in kinetic mode every 5 minutes for 30 min at 37 °C (see SOP 4b, paragraph 3 for details [9]) and the concentrations of methylumbelliferone were calculated by comparison with calibration curve. Quality control and blank control were also added to the reading plate and prepared as described in SOP 4b, paragraph 3 [9]).

7.2.1 Analysis of MU fluorescence assay

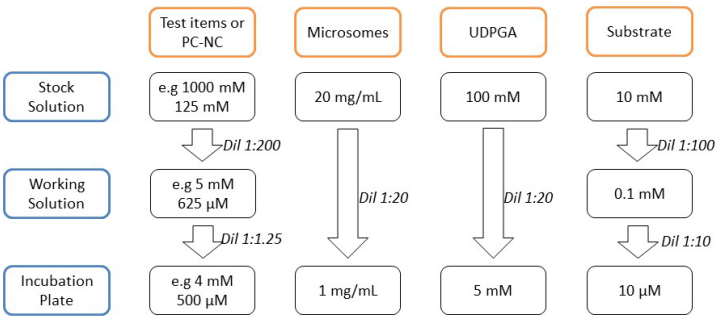
UGTs activity was determined by measuring the disappearance of methylumbelliferone, a fluorescent substance transformed into non-fluorescent methylumbelliferone glucuronide.

The incubation and analytical samples (control blank, calibration curve, quality controls) were prepared and analysed as described in paragraph 3 of SOP 4b [9].

7.3 Inhibition Assay

The inhibition of UGTs activity was initially evaluated in a "range finding assay" where the test item was tested at eight concentrations within a wide range in order to identify the ideal concentration range of the "main inhibition assay" where the IC50 values of the test item were determined in 5 valid runs.

The incubation assay was performed was performed with automated liquid handling system (Multiprobe II System, Perkin Elmer) in 96-well plates using the concentrations and the volumes described in the following figure:



Incubation Plate	Dilution	Volume
Test Item, PC, NC or solvent control WS	1:1.25	200 µL
Microsomes	1:20	12.5 µL
UDPGA	1:20	12.5 µL
Substrates	1:10	25 µL
Total Volume		250 µL

The following treatment groups were included:

- "Blank with test item" consisting of human liver microsomes, incubation medium, substrates (T3 or T4) and the test item at the highest concentration (UDPGA cofactor was not included). These controls were evaluated to ensure that the test item in the system didn't cause analytical interferences and to aid calculation of UGT activity in the MU assay (column 1).

- Test item at eight different concentrations (columns 2-9, where C1 is the highest concentration).
- Negative control (NC) at one specific concentration (column 9)
- Solvent-control consisting of all incubation components and 0.5% DMSO (column 11).
- No solvent-control consisting of incubation medium, HLM 1 mg/mL and UDPGA. This control was used to check the effect of the solvent on the enzymatic reaction (column 12).

Different concentrations of substrates were placed in different rows: T3 2 µM and T3 5 µM in rows 2-4 and 5-7 of incubation plate 1, T3 10 µM and T4 2 µM in rows 2-4 and 5-7 of incubation plate 2, T4 5 µM and T4 10 µM in rows 2-4 and 5-7 of incubation plate 3. Rows A and H were filled with water and were not analysed.

Incubation plate 1:

	1	2	3	4	5	6	7	8	9	10	11	12
A	w	w	w	w	w	w	w	w	w	w	w	w
B	TI C1 No UDPGA T3 2 µM	TI C1 T3 2 µM	TI C2 T3 2 µM	TI C3 T3 2 µM	TI C4 T3 2 µM	TI C5 T3 2 µM	TI C6 T3 2 µM	TI C7 T3 2 µM	TI C8 T3 2 µM	NC C1 T3 2 µM	Solvent T3 2 µM	No Solvent T3 2 µM
C	TI C1 No UDPGA T3 2 µM	TI C1 T3 2 µM	TI C2 T3 2 µM	TI C3 T3 2 µM	TI C4 T3 2 µM	TI C5 T3 2 µM	TI C6 T3 2 µM	TI C7 T3 2 µM	TI C8 T3 2 µM	NC C1 T3 2 µM	Solvent T3 2 µM	No Solvent T3 2 µM
D	TI C1 No UDPGA T3 2 µM	TI C1 T3 2 µM	TI C2 T3 2 µM	TI C3 T3 2 µM	TI C4 T3 2 µM	TI C5 T3 2 µM	TI C6 T3 2 µM	TI C7 T3 2 µM	TI C8 T3 2 µM	NC C1 T3 2 µM	Solvent T3 2 µM	No Solvent T3 2 µM
E	TI C1 No UDPGA T3 5 µM	TI C1 T3 5 µM	TI C2 T3 5 µM	TI C3 T3 5 µM	TI C4 T3 5 µM	TI C5 T3 5 µM	TI C6 T3 5 µM	TI C7 T3 5 µM	TI C8 T3 5 µM	NC C1 T3 5 µM	Solvent T3 5 µM	No Solvent T3 5 µM
F	TI C1 No UDPGA T3 5 µM	TI C1 T3 5 µM	TI C2 T3 5 µM	TI C3 T3 5 µM	TI C4 T3 5 µM	TI C5 T3 5 µM	TI C6 T3 5 µM	TI C7 T3 5 µM	TI C8 T3 5 µM	NC C1 T3 5 µM	Solvent T3 5 µM	No Solvent T3 5 µM
G	TI C1 No UDPGA T3 5 µM	TI C1 T3 5 µM	TI C2 T3 5 µM	TI C3 T3 5 µM	TI C4 T3 5 µM	TI C5 T3 5 µM	TI C6 T3 5 µM	TI C7 T3 5 µM	TI C8 T3 5 µM	NC C1 T3 5 µM	Solvent T3 5 µM	No Solvent T3 5 µM
H	w	w	w	w	w	w	w	w	w	w	w	w

Incubation plate 2:

	1	2	3	4	5	6	7	8	9	10	11	12
A	w	w	w	w	w	w	w	w	w	w	w	w
B	TI C1 No UDPGA T3 10 µM	TI C1 T3 10 µM	TI C2 T3 10 µM	TI C3 T3 10 µM	TI C4 T3 10 µM	TI C5 T3 10 µM	TI C6 T3 10 µM	TI C7 T3 10 µM	TI C8 T3 10 µM	NC C1 T3 10 µM	Solvent T3 10 µM	No Solvent T3 10 µM
C	TI C1 No UDPGA T3 10 µM	TI C1 T3 10 µM	TI C2 T3 10 µM	TI C3 T3 10 µM	TI C4 T3 10 µM	TI C5 T3 10 µM	TI C6 T3 10 µM	TI C7 T3 10 µM	TI C8 T3 10 µM	NC C1 T3 10 µM	Solvent T3 10 µM	No Solvent T3 10 µM
D	TI C1 No UDPGA T3 10 µM	TI C1 T3 10 µM	TI C2 T3 10 µM	TI C3 T3 10 µM	TI C4 T3 10 µM	TI C5 T3 10 µM	TI C6 T3 10 µM	TI C7 T3 10 µM	TI C8 T3 10 µM	NC C1 T3 10 µM	Solvent T3 10 µM	No Solvent T3 10 µM
E	TI C1 No UDPGA T4 2 µM	TI C1 T4 2 µM	TI C2 T4 2 µM	TI C3 T4 2 µM	TI C4 T4 2 µM	TI C5 T4 2 µM	TI C6 T4 2 µM	TI C7 T4 2 µM	TI C8 T4 2 µM	NC C1 T4 2 µM	Solvent T4 2 µM	No Solvent T4 2 µM
F	TI C1 No UDPGA T4 2 µM	TI C1 T4 2 µM	TI C2 T4 2 µM	TI C3 T4 2 µM	TI C4 T4 2 µM	TI C5 T4 2 µM	TI C6 T4 2 µM	TI C7 T4 2 µM	TI C8 T4 2 µM	NC C1 T4 2 µM	Solvent T4 2 µM	No Solvent T4 2 µM
G	TI C1 No UDPGA T4 2 µM	TI C1 T4 2 µM	TI C2 T4 2 µM	TI C3 T4 2 µM	TI C4 T4 2 µM	TI C5 T4 2 µM	TI C6 T4 2 µM	TI C7 T4 2 µM	TI C8 T4 2 µM	NC C1 T4 2 µM	Solvent T4 2 µM	No Solvent T4 2 µM
H	w	w	w	w	w	w	w	w	w	w	w	w

Incubation plate 3:

	1	2	3	4	5	6	7	8	9	10	11	12
A	w	w	w	w	w	w	w	w	w	w	w	w
B	TI C1 No UDPGA T4 5 µM	TI C1 T4 5 µM	TI C2 T4 5 µM	TI C3 T4 5 µM	TI C4 T4 5 µM	TI C5 T4 5 µM	TI C6 T4 5 µM	TI C7 T4 5 µM	TI C8 T4 5 µM	NC C1 T4 5 µM	Solvent T4 5 µM	No Solvent T4 5 µM
C	TI C1 No UDPGA T4 5 µM	TI C1 T4 5 µM	TI C2 T4 5 µM	TI C3 T4 5 µM	TI C4 T4 5 µM	TI C5 T4 5 µM	TI C6 T4 5 µM	TI C7 T4 5 µM	TI C8 T4 5 µM	NC C1 T4 5 µM	Solvent T4 5 µM	No Solvent T4 5 µM
D	TI C1 No UDPGA T4 5 µM	TI C1 T4 5 µM	TI C2 T4 5 µM	TI C3 T4 5 µM	TI C4 T4 5 µM	TI C5 T4 5 µM	TI C6 T4 5 µM	TI C7 T4 5 µM	TI C8 T4 5 µM	NC C1 T4 5 µM	Solvent T4 5 µM	No Solvent T4 5 µM
E	TI C1 No UDPGA T4 10 µM	TI C1 T4 10 µM	TI C2 T4 10 µM	TI C3 T4 10 µM	TI C4 T4 10 µM	TI C5 T4 10 µM	TI C6 T4 10 µM	TI C7 T4 10 µM	TI C8 T4 10 µM	NC C1 T4 10 µM	Solvent T4 10 µM	No Solvent T4 10 µM
F	TI C1 No UDPGA T4 10 µM	TI C1 T4 10 µM	TI C2 T4 10 µM	TI C3 T4 10 µM	TI C4 T4 10 µM	TI C5 T4 10 µM	TI C6 T4 10 µM	TI C7 T4 10 µM	TI C8 T4 10 µM	NC C1 T4 10 µM	Solvent T4 10 µM	No Solvent T4 10 µM
G	TI C1 No UDPGA T4 10 µM	TI C1 T4 10 µM	TI C2 T4 10 µM	TI C3 T4 10 µM	TI C4 T4 10 µM	TI C5 T4 10 µM	TI C6 T4 10 µM	TI C7 T4 10 µM	TI C8 T4 10 µM	NC C1 T4 10 µM	Solvent T4 10 µM	No Solvent T4 10 µM
H	w	w	w	w	w	w	w	w	w	w	w	w

Abbreviations: TI = Test Item; C1-C8= Concentrations; Solvent = Solvent control; NC = Negative Control; w = water

7.3.1 Inhibition enzyme activity assay: Assay Procedure

The determination of UGTs inhibition was described in detail in paragraph 2.7.3 and 2.7.4 of SOP No. 4b [9].

Test item , Solvent-Control, No solvent Control Working solution were thawed, vortexed an alamethicin solution at 50 mg/mL (see paragraph 5) added in order to obtain a final concentration of 25 µg/mL. Solutions were vortexed, sonicated for 15 minutes at 40°C and then dispensed in the incubation plates.

Microsomes were thawed and diluted to 20 mg/mL (paragraph 2.3.4.1 of SOP method 4b [9]) and dispensed in the incubation plates (final concentration of 1 mg/mL). A pre-incubation of 15 ± 5 min was performed to aid pore-forming activity of alamethicin.

UDPGA solution was prepared as described in 4.4.2 and dispensed in all wells at 5 mM except in column 1.

T3 and T4 stock solutions were thawed, vortexed and diluted in water as described in paragraph 5.1. These working solutions were dispensed quickly (column by column) in all the wells. The addition of the substrates to the first well was recorded as the starting incubation time.

At different time points (20 minutes for incubation plate 1, 60 minutes for plate 2 and 120 minutes for plate 3), the incubation solutions were transferred into the reading plate, diluted 5-fold with the same working solution and incubated with methylumbelliferone (80 µM). These plates were immediately read at the plate reader fluorometer. Fluorescence was measured at Ex/Em = 372/445 nm in kinetic mode every 5 minutes for 30 min at 37 °C (see SOP 4b, paragraph 3 for details, [9]) and the concentrations of methylumbelliferone calculated by comparison with the calibration curve. Quality control and blank control were also added in the reading plate and prepared as described in the (SOP 4b, paragraph 3 [9]).

After 180 minutes, the incubation solutions were transferred into two 384 well plates containing the same volume of stopping solution (paragraph 4.4.6). These plates were subsequently centrifuged (15 min at ≥ 2,200 g), and one was analysed (analytical plate) and the other immediately frozen at –20 °C (backup plate).

7.3.2 Range Finding Assay

The range finding assay was performed in one run in order to estimate the inhibition behavior of test item and determine the estimated IC50 curve. The concentrations of stock and working solutions are described in paragraphs 5.2.1 and 5.2.2, respectively.

7.3.3 Main inhibition Assay

The estimated IC50 curve evaluated in the range finding assay was used to evaluate the inhibition activity of test item. New concentrations were chosen for the main inhibition assay performed in five valid runs. The stock and working solutions preparation of the test item are described in paragraphs 5.2.3 and 5.2.4, respectively.

7.3.4 Analysis of inhibition Assay

The inhibition of UGTs activity of mefenamic acid was quantified by measuring the formation of T3 and T4 glucuronides.

The incubation and analytical samples (control blank, calibration curve, quality controls) were prepared and analysed as described in the paragraph 4 of SOP 4b [9]).

7.4 Calculation of Results

7.4.1 Test Item Concentration Check

The parameter calculated and formulae are described in detail in the analytical procedure [6] and summarized in the following tables:

Item	Formula
Reference Standard precision	$\%CV = \frac{Reference\ Standard\ Deviation}{Area\ Average} x100$
Accuracy of sample solution	$\%Assay = \frac{Area\ of\ sample\ x\ Initial\ Volume}{RF\ x\ Final\ volume\ (mL)\ x\ Nominal\ Concentration} x100$ $Where\ RF = \frac{Reference\ Standard\ Area}{Refrence\ Standard\ Cocnetration}$
Precision of sample solution	$\%CV = \frac{Area\ Sample\ Deviation}{Area\ Sample\ Average} x100$

7.4.2 Substrates Concentration Check

The concentration micromolar of T3 and T4 were calculated with Analyst software comparing the normalized data (ratio between area of analyte and area of ISTD) against the calibration curve. Also, the accuracy of calibration standard and parameters were calculated with Analyst software.

The parameter calculated and formulae are described in detail in the paragraph 4.2 of SOP 4b [9] and summarized in the following tables:

Item	Formulae
Intra-run precision of sample solution	$\%CV(intra-run) = \frac{Standard\ Deviation\ of\ Triplicate}{Mean\ of\ Triplicate} x100$

7.4.3 Characterization of UGTs Activity: MU fluorescence Assay

The micromolar concentration of MU were calculated with Microsoft Excel software. The choice of time to calculate the UGTs activity was important for reproducibility: RFU_1 must be <20 000 and RFU_2 after 10 - 15 min.

Calculations are described in detail in paragraph 3.4 of SOP 4b [9] and summarized in the following tables:

Item	Formula
UGTs activity [mU/mg]	$\Delta F = \lvert RFU_2 - RFU_1 \rvert \rightarrow G_S = \Delta F_S - \Delta F_{no\ UDPGA} \rightarrow$ UGTs Activity $= \frac{B}{\Delta T \times P}$ (where B is the nmole of MU consumed, ΔT is the time and P the amount of HLM)
Percentage of inhibition	$\%\ inhibition = 100 - \frac{\mu M\ metablite\ in\ presence\ of TI\ or\ NC}{\mu M\ product\ in\ solvent\ control} x100$
Intra-plate precision	$\%CV(intra-plate) = \frac{Standard\ Deviation\ of\ Triplicate}{Mean\ of\ Triplicate} x100$
Inter –plate precision	$\%\ CV(inter\ plate) = \frac{Standard\ Deviation\ of\ mean\ intra\ plate}{Mean\ intra\ plate} x100$
Inter-run precision:	$\%\ CV(inter\ run) = \frac{Standard\ Deviation\ of\ mean\ inter\ plate}{Mean\ inter\ plate} x100$

7.4.4 Inhibition assay

The concentration micromolar of T3G and T4G were calculated with Analyst software comparing the normalized data (ratio between area of analyte and area of ISTD) against the calibration curve. Also, the accuracy of calibration standard and parameters were calculated with Analyst software

The parameter calculated and formulae are described in detail in the paragraph 4.2 of SOP 4b and summarized in the following tables:

Item	Formulae
Glucuronide formation (solvent control)	$Formation\ Rate\ = \frac{\mu M\ product\ x\ 1000}{min\ of\ incubation\ x\ 1\ mg/mL}$
Percentage of inhibition	$\%\ inhibition\ = 100 - \frac{\mu M\ metablite\ in\ presence\ of TI\ or\ NC}{\mu M\ product\ in\ solvent\ control}\ x100$
Percentage of activity vs control solvent	$\%\ activity = 100 - \frac{\mu M\ metablite\ in\ presence\ of TI, NC\ or\ No\ solvent}{\mu M\ product\ in\ solvent\ control}\ x100$
IC50	$IC50\ = \frac{Bottom + (Top - Bottom)}{1 + 10\text{^}((logIC50 - X)\ x\ HillSlope)}$
Intra-plate precision	$\%CV(intra - plate)\ = \frac{Standard\ Deviation\ of\ Triplicate}{Mean\ of\ Triplicate}\ x100$
Inter-plate precision	$\%\ CV(inter\ plate)\ = \frac{Standard\ Deviation\ of\ mean\ intra\ plate}{Mean\ intra\ plate}\ x100$
Inter-run precision:	$\%\ CV(inter\ run)\ = \frac{Standard\ Deviation\ of\ mean\ inter\ plate}{Mean\ inter\ plate}\ x100$

7.5 Acceptance criteria applied

7.5.1 Concentration check of substrates and test item

The acceptance criteria of the concentration check of test item:

Validation Item	Minimum experiments	Default Acceptance Criteria
Reference Standard Solutions	Perform 6 (six) replicate injections for Reference Standard Solution containing Mefenamic Acid	Peak Area: CV % ≤ 5.0 %
Test Item WS: Accuracy and precision	Solution of Mefenamic Acid; n=3 for each solution	Assay % = 85 – 115 % CV % ≤ 15.0 %

7.5.2 Substrates Concentration Check

The acceptance criteria of the concentration check of substrates:

Validation Item	Minimum experiments	Default Acceptance Criteria
Calibration Standards:	8 non-zero concentrations in duplicate	Minimum 75% of the CS should be within ±15.0% of target concentrations except at LLOQ ±20.0%. R-squared ≥0.990
Substrates SS: Accuracy and Precision	Solution of T3 and T4; n=3 for each solution	CV % ≤ 15% Assay % ≤ ±15 %

7.5.3 Characterization of UGTs activity of the test system: MU fluorescence

The new batch of microsomes (QQY) was described and characterized by the provider (BioIVT) and values reported in the Certificate of analysis. Some values are considered as acceptance criteria:

Validation Item	Minimum experiments	Default Acceptance Criteria
HLM	Protein concentration Donors	≥ 20 mg/mL ≥ 50 donors
Rate of glucuronide formation of HLM	In the CoA: UGT1 or UGT1A1 rate of formation	> 900 pmol/min/mg

The UGTs activity of QQY were determined by MU fluorescence assay. The acceptance criteria of methylumbelliferone analytical runs are summarized in the following table:

Validation Item	Minimum experiments	Default Acceptance Criteria
Blank control and Blank control with PC/NC	Signal-to-noise of incubation matrix or PC/NC without MU	≤ LLOQ
Calibration Curve	At least 6 non-zero concentrations in duplicate	Minimum 75% of the STD should be considered. 80 -120 % except LLOQ and ULOQ 75 – 125 % $R^2 \geq 0.990$
QC Samples: Accuracy and precision	3 concentrations: low QC (LQC), mid QC (MQC), high QC (HQC), n=2 in each run	Mean intra- accuracy 80 – 120 %, and

The acceptance criteria related to UGTs activity and inhibition by MU fluorescence assay were:

Validation Item	Minimum experiments	Default Acceptance Criteria
UGTs activity	In no solvent-control	≥ 80 mU/mg
Test Item Control Inhibition	Percentage of Mefenamic Acid inhibition at highest Conc.	> 60%
Negative Control Inhibition	Percentage of Fluconazole inhibition	< 20%
Precision intra and inter-plates	3 replicates for each sample: Average of each run of Solvent-control, No Solvent-control, PC, NC (%CV)	≤ 20% intra-plate ≤ 20% inter-plate (if there are multiple plates)
Precision and accuracy inter-run	Average of each run vs new batch assay in the Solvent-control, no solvent-control (CV% and Bias%)	≤ 20% CV% inter-run ≤ ±20% Bias% inter-run

7.5.4 Inhibition assay

The acceptance criteria of the analytical runs in the inhibition assay are summarized in the following table:

Validation Item	Minimum experiments	Default Acceptance Criteria
Matrix (Background)	Signal-to-noise co-eluting with-metabolite Signal-to-noise co-eluting with-ISTD	≤20% vs LLOQ ≤5% vs ISTD
Calibration Standards:	At least 6 non-zero concentrations in duplicate	Minimum 75% of the CS should be within ±15.0% of target concentrations except at LLOQ ±20.0%. CV% intra-run ≤ 20%
QC Samples: Accuracy and precision	3 concentrations: low QC (LQC), mid QC (MQC), high QC (HQC), n=2 in each run	Mean intra- accuracy 85.0-115.0%, and CV ≤ +15.0%

The acceptance criteria of T3 and T4 glucuronide inhibition were the following:

Validation Item	Minimum experiments	Default Acceptance Criteria
Amount of glucuronide conjugates without cofactor	Signal-to-noise co-eluting with-metabolite Signal-to-noise co-eluting with-ISTD	≤ LLOQ ≤5% vs ISTD
Rate Formation of glucuronide conjugate in solvent control	Rate Formation of glucuronide with incubation of T3 and T4 10 µM	> 0.40 pmol/mg protein/min (T3G) > 2.00 pmol/mg protein/min (T4G)
Intra-run and Inter - run Precision	3 replicates for each sample % activity versus solvent	CV ≤ 20.0%
Effect of solvent	Amount of glucuronide in the no-solvent control versus solvent	≤ ± 20%
Test Item Control Inhibition	Percentage of Mefenamic Acid inhibition at highest Conc.	> 60%
Negative Control Inhibition	Percentage of Fluconazole inhibition	< 20%
IC50 curve	Intra-run: 8 concentration point of mefenamic acid in triplicate Inter-run: 8 concentrations point of mefenamic acid of 5 validated runs Inter-run precision	R-square ≥ 0.9000. Minimum 75% of the points should be accepted (intra-run n≥20, inter run n≥33) CV ≤ 20.0%

7.6 Deviations

In the “range finding assay” T4G quality controls were outside of acceptance criteria: only three (instead of at least four) out of 6 showed an intra-run accuracy ≤ 15%. Moreover, in the same assay, T3 and T4 were incubated twice at a concentration of 5 µM instead of 5 µM. The experiment was not rejected as it was intended just to estimate the proper concentration range of mefenamic acid and data were not part of the required five valid runs.

In run 4 of the main inhibition assay (22 July 202) when T3 and T4 were incubated with no solvent and negative controls the percentages activity versus solvent were out of the acceptance range, 80-120% (data are not shown in this report but are documented in the raw data files). It is likely that alamethicin was not added to the working solution resulting in less glucuronide formation. Based on the results obtained in the other

validated runs and in the preliminary data, the no solvent samples were used to determine percentage of activity and inhibition of the other conditions, as the presence of the solvent did not have any effect on glucuronide formation (% activity of no solvent versus % activity of solvent only is in the range 80-120%).

8 RESULTS AND EVALUATION

The robustness and reliability of the inhibition of T3 and T4 glucuronides formation was assessed in this study by performing five valid runs using mefenamic acid as test item. These studies were performed in different steps and different days as reported in Table 1.

8.1 Concentration check of test item

8.1.1 Concentration check of test item: Reference Standards precision

Two Reference Standard Solutions of Mefenamic Acid were injected in triplicate at 0.100 µM in two analytical runs. The precision was 4.4 and 4.9%, within the tolerance limits (CV% ≤ 5.0%). Data reported in Table 2.

8.1.2 Concentration check of test item: Working solutions accuracy and precision

Accuracy and precision of mefenamic acid working solutions were evaluated with three injections and expressed as Mean Assay % and CV%.

Mean Assay % and CV% values were within the acceptable range 85-115 % and % ≤ 15.0% as reported in Table 3 (WS of the range finding assay) and Table 4 (WS of the main inhibition assay)

8.2 Concentration check of substrates:

8.2.1 Concentration check of substrates: Linearity

A summary of the back-calculated concentrations for calibration points and calibration line parameters obtained are reported in Table 5 and Table 6 for T3G and Table 8 and Table 9 for T4G, respectively. The lower and upper limits of quantitation were 0.094 and 12.000 µM, respectively. The coefficient of determination (r^2) were 0.9973 for T3G and 0.9948 for T4G. The mean back-calculated concentration values showed an accuracy (expressed as % bias) ranging from -5.1 to 8.2 % for T3G and -14.4 to 14.0 %.

8.2.2 Concentration check of substrates: Stock solutions accuracy and precision

The accuracy and precision of T3 and T4 sock solutions at 2, 5 and 10 mM were evaluated with three injections and expressed as Mean Assay % and CV%.

Mean Assay % and CV% values of T3 and T4 stock solutions were within the acceptable range 85-115 % and % ≤ 15.0% as reported in Table 7 and Table 10, respectively.

8.3 Methylumbelliferone Fluorescence Assay: Characterization of UGTs activity of HLM

8.3.1 Fitting of the Calibration Curve

A summary of the back-calculated concentrations for calibration curve parameters and the calibration standards are reported in Table 11. Only runs with acceptable results obtained by applying the same experimental final conditions were included. The coefficient of determination (r^2), ranged from 0.9988 to 0.9998. The 1/Y weighing factor was selected to minimize the deviations of the back-calculated values from their nominal concentrations.

8.3.2 Accuracy and Precision of Methylumbelliferone Quality Controls

The results for the intra-plate, inter-plate and inter-run precision and accuracy of the methylumbelliferone quality controls samples at 2.5, 10 and 60 µM are shown in Table 12. The intra-plate and inter-plate accuracy (expressed as Bias %) ranged from -18.8 to 19.6% and -13.7 to 17.7%, respectively. Inter-run precision (expressed as %CV) and accuracy ranged from 3.3 to 5.7% and from -8.9 to 12.4%, respectively.

8.3.3 Characterization of UGTs activity of new HLM batch

The UGTs activity of QQY HLM batch was determined by the methylumbelliferone assay in two valid runs and it is reported in Table 13. The UGTs activity values of solvent and no-solvent controls were within the acceptance criteria (≥ 80 mU/mg) and ranged from 93.2 to 134.3 mU/mg.

Intra-run and inter-run precision (expressed as CV%) ranged from 0.3 to 16.2% and from 0.3 to 1.2%, respectively.

8.3.4 Characterization of UGTs activity of new HLM batch in parallel with inhibition assay

The UGTs activity of QQY HLM batch was determined via the methylumbelliferone assay in all runs (range finding assay and main inhibition assay), as reported in Table 14.

Intra-run mean UGT activity in the solvent and no-solvent controls were always ≥ 80 mU/mg (ranged from 97.1 to 116.1 mU/mg). Intra-run precision and accuracy ranged from 4.5 to 18.5 and from -14.4 to 8.0%, respectively.

Inter-run UGT activity mean values of solvent and no solvent control were 111.3 and 110.2 mU/mg, respectively. Inter-run precision and accuracy of both conditions were within the acceptance criteria (CV% 7.9 and 6.8 and Bias% -2.6 and -10.3%, respectively).

8.3.5 Evaluation of percentage inhibition via Methylumbelliferone fluorescence assay in the valid runs

The percentages of inhibition of test item, negative control and no-solvent control were determined via the methylumbelliferone assay in all runs (range finding assay and main inhibition assay), as reported in Table 15. The inhibition was calculated versus solvent control.

The intra-run average percentages of mefenamic acid at highest concentration (500 µM), ranged from 68.7 to 74.6% (AC ≥ 60.0 %). No inhibition was observed in the presence of the negative control, fluconazole at 500 µM (% inhibition ranged from -1.6 to 3.9) and in the no-solvent control (from -3.0 to 8.3): these percentages of inhibition were within the acceptance criteria (AC ≤ 20.0 %).

8.4 Inhibition Assay

8.4.1 Linearity of T3G and T4G

A summary of the back-calculated concentrations for the calibration points and calibration curve parameters obtained are reported in Table 16 and Table 17 for T3G and Table 31 and Table 32 for T4G, respectively. The lower and upper limits of quantitation were 0.005 and 2.000 µM, respectively. The coefficient of determination (r^2) ranged from 0.9922 to 0.9969 for T3G and 0.9919 to 0.9973 for T4G. The inter-run mean back-calculated concentration values showed an accuracy (expressed as bias %) ranging from

-4.2 to 5.3 % for T3G, -8.1 to 6.7 % for T4G and a precision (expressed as CV%) ranging from 6.9 to 12.9% for T3G and from 7.1 to 10.9% for T4G.

8.4.2 Inter and intra-assay precision and accuracy of T3G and T4G Quality Controls

The results for the intra- and inter-assay precision and accuracy are shown in Table 18 for T3G and in Table 33 for T4G quality controls. The intra-assay accuracy ranged from -18.1 to 14.7 % (T3G) and from -14.2 to 18.0 % (T4G) and the inter-assay accuracy ranged from -0.3 to 8.3 % (T3G) and from -5.4 to 12.6% (T4G). The inter-assay precision (expressed as %CV) ranged from 9.1 to 11.4% (T3G) and from 8.4 to 10.7% (T4G).

The T4G quality controls of the range finding assay were outside the acceptance criteria: only three quality controls (of the required 4) were accepted with intra-assay accuracy ≤ 15% (see paragraph 7.6).

8.4.3 Selectivity and no cofactor incubation

The incubation matrix was analysed in order to evaluate possible interferences (Table 19 and Table 34). These samples were prepared with fixed concentration of T3 or T4 (10 µM) in presence of ISTD at the concentration usually used for the assay (1 µM). In all valid runs no peak at the T3G or T4G retention times (3.7 and 2.9 minutes, respectively) was observed.

No interference was observed also when T3 and T4 10 µM were incubated for 3 hours with human liver microsomes without cofactor UDPGA (Table 20 and Table 35): when the UGT enzyme was inactive due to the absence of the cofactor, the formation of glucuronides was not observed

8.4.4 Range finding Assay: effect of Mefenamic Acid and No Solvent on the glucuronides' formation

The inhibition of Mefenamic Acid was evaluated at eight different concentrations (500, 200, 50, 20, 5, 2, 0.2 and 0.05 µM) for 3 hours. T3 and T4 were incubated at 5 and 10 µM (5 µM was not incubated, see paragraph 7.6). The intra-run precision of percentages of activity were within the acceptance criteria (≤ 20%). Mefenamic acid inhibited T3G (Table 21) and T4G (Table 36) at higher concentration than 50 µM, while at lower concentrations the percentage of inhibition was less than 20% (five out of eight concentrations). Fluconazole at 500 µM didn't inhibit T3G and T4G formation.

8.4.5 Main inhibition Assay: glucuronide formation in the "Solvent" in the five valid runs

The formation of T3 and T4 glucuronides was calculated following 180 minutes of incubation with 2, 5 and 10 µM of T3 or T4 in Solvent Control (incubation matrix with 0.5% DMSO). Table 22 and Table 37 show the concentrations and the rate of formation of glucuronides in the five valid runs performed in triplicate. The intra-precision values ranged from 2.2 to 19.7% for T3G and from 2.1 to 17.1% for T4G. The glucuronides formation increased with increasing concentration of substrate: inter-run means of T3G formation were 0.053, 0.120 and 0.201 µM when T3 were incubated at 2, 5 and 10 µM. While the T4G formation was higher, with inter-run means 0.208, 0.512 and 1.200 µM when T4 was incubated at 2, 5 and 10 µM. These inter-run means were calculated

excluding the intra-run of run 4 performed on 22 July 2021 because the formation of T3 and T4 glucuronide concentrations were lower than other valid runs (see paragraph 7.6).

8.4.6 Main inhibition Assay: glucuronide formation in the "No Solvent and Negative Control" in the five valid runs

The formation of T3 and T4 glucuronides was calculated at 180 minutes of incubation with 2, 5 and 10 µM of T3 or T4 in No Solvent Control (incubation matrix only) and Negative Control (Fluconazole 500 µM).Table 23, Table 24, Table 38 and Table 39 show the glucuronides concentrations and the percentage of activity in the five valid runs performed in triplicate. Intra-run and inter-run precision values were within the acceptance criteria (≤ 20%). The percentages of activity versus solvent were within 80-120% when T3 or T4 at 2, 5 and 10 µM were incubated with no solvent: 0.5% had no effect on T3G and T4G formation. The same results were obtained with fluconazole, confirming that it can be considered a proper negative control.

In the run 4 performed on 22 July 2021 the percentages of activity were calculated versus solvent control (see paragraph 7.6).

8.4.7 Main Inhibition Assay: glucuronides formation in the Test Item in the five valid runs

The formation of T3 and T4 glucuronides was calculated at 180 minutes of incubation with 2, 5 and 10 µM of T3 (Table 26, Table 27 and Table 28, respectively) or T4 (Table 40, Table 41 and Table 42) in the presence of the test item. The percentages of activity versus solvent of mefenamic acid at eight concentrations (500, 320, 200, 160, 80, 50, 20 and 5 µM) were evaluated in five valid runs. Intra-run and inter-run precision values were within the acceptance criteria (≤ 20%). The percentages activity increased with increasing mefenamic acid concentrations.

In the run 4 performed on 22 July 2021 the percentages of activity were calculated versus solvent control (see paragraph 7.6).

8.4.8 Main Inhibition Assay: IC50 Curve of glucuronides in presence of Test Item in the five valid runs

The IC50 curves were drawn using GraphPad Software. Curves represent the percentage of activity versus solvent (Y-axis) at different concentration of mefenamic acid (X-axis) when substrates T3 (Table 28) and T4 (Table 43) were incubated at 2, 5, and 10 µM. The R-square were reported from 0.9112 to 0.9683 for T3G curves and from 0.9305 to 0.9880 for T4G curves. The IC50 intra-run were calculated using the percentages activity of each valid runs (in triplicate). The inter-run IC50 curves were the average of the five IC50 intra-run average calculated in each valid runs. The inter-run IC50 of T3G were 57.86, 77.88 and 142.66 µM at 2, 5 and 10 µM of T3, respectively, and 96.05, 97.66 and 109.51 µM for T4G at 2, 5 and 10 µM of T4, respectively. The inter-run precision of IC50 of T3G (Table 30) and of T4G (Table 45) were ≤ 20%.

IC50 inter-run values were similar when they were calculated by the intra-run average of activy percentage (57.42, 79.52 and 144.1 µM for T3G at 2, 5 and 10 µM of T3 and 93.74, 97.15 and 106.9 µM for T4G at 2, 5 and 10 µM of T4).

8.4.9 Main Inhibition Assay: summary of inhibition of glucuronides formation

The percentages of inhibition versus solvent were calculated in each valid run by incubating T3 (Table 29) and T4 (Table 44) at 2, 5 and 10 µM with different

concentration of mefenamic acid (500, 320, 200, 160, 80, 50, 20 and 5 µM). In the run 4 performed on 22 July 2021 the percentages of inhibition were calculated versus solvent control (see paragraph 7.6).

The percent inhibition increased with increasing mefenamic acid concentrations. The inhibition of T3G with equal concentrations of mefenamic acid was lower at higher substrate's concentration. (e.g. at 200 µM mefenamic acid, it was 82.0, 74.3 and 57.6 µM with 2, 5 and 10 µM of T3, respectively). On the contrary, inhibition of T4G didn't change by varying substrate's concentration (e.g. at 200 µM mefenamic acid, it was 65.9, 67.7 and 65.5 µM with 2, 5 and 10 µM of T4, respectively).

At the highest concentration of mefenamic acid the percentages of inhibition were within the acceptance criteria (≥ 60%).

9 CONCLUSION/DISCUSSION

The inhibition of glucuronidation of T3 and T4 was evaluated in five valid runs.

Based on the results obtained, the analytical method of T3G and T4G demonstrated adequate selectivity, specificity, sensitivity, carry-over, linearity, precision and accuracy over the nominal range of 0.005 – 2.000 µM. Furthermore, the study confirmed the robustness and the reliability of method 4b based on the related standard operating procedure (SOP No. 4b). As required, all acceptance criteria were met in five valid runs and the selected positive and negative controls (mefenamic acid and fluconazole, respectively) were confirmed to be proper controls for part 2 of the process.

10 RECORDS TO BE RETAINED

A copy of the report as original and supporting documents, are filed in the Archives of Accelera S.r.l., Nerviano (Italy) for the period up to 3 years after which EURL ECVAM will be contacted for instructions regarding dispatch or disposal of the material. A copy of this report with scanned signatures is sent to the EURL ECVAM. The final report in PDF format sent to EURL ECVAM totally corresponds to the full paper copy of the original final report. All raw data in electronic form will send to EURL ECVAM.

11 QUALITY ASSURANCE

This study will not be audited by QA. As appropriate, the Standard Operating Procedures of the laboratories involved will be followed in this study as well as the OECD Principles of GLP and OECD Guidance Document on Good In Vitro Method Practices (GIVIMP).

12 TABLES

12.1 Tables: Study design and test items

Table 1 Summary of Part 1 Experiments

N° ID	Assay	Run	Regression Status	Assay Date	Experimental Content
1	Concentration Check: Test item	N/A	Accepted	17 June 2021	TI, NC, Solvent and No Solvent Control SS and WS
2	Preparation of MU QC	N/A	Accepted	21 Jun 2021	MU QC and SS Preparations and Concentration check
3	MU Fluorescence Assay of New HLM Batch	RUN 1	Accepted	23 Jun 2021	HLM characterization of UGTs activities
4	MU Fluorescence Assay of New HLM Batch	RUN 2	Accepted	24 Jun 2021	HLM characterization of UGTs activities
5	Preparation of T3G QC	N/A	Accepted	29 Jun 2021	T3G QC Preparations and Concentration check
6	Preparation of Fexofenadine solutions	N/A	Accepted	29 Jun 2021	Fexofenadine WS Preparations and Concentration check
7	Preparation of T4G QC	N/A	Accepted	30 Jun 2021	T4G QC Preparations and Concentration check
8	Concentration Check: substrates	N/A	Accepted	01 July 2021	T3 and T4 stock SS Preparations and Concentration check
9	Range Finding Assay	N/A	Accepted[(a)]	01 July 2021	Inhibition Assay of T3G and T4G formation: evaluation of mefenamic acid concentrations. In parallel MU fluorescence assay
10	Concentration Check: test item	N/A	Accepted	05 July 2021	Test items and WS Preparation and Concentration Check
11	Main Inhibition Assay	RUN 1	Accepted	06 July 2021	Inhibition Assay of mefenamic acid at chosen concentrations on the T3G and T4G formatios. In parallel MU fluorescence
12	Main Inhibition Assay	RUN 2	Accepted	09 July 2021	
13	Main Inhibition Assay	RUN 3	Accepted	14 July 2021	
14	Main Inhibition Assay	RUN 4	Accepted[(a)]	22 July 2021	
15	Main Inhibition Assay	RUN 5	Accepted	05 August 2021	

[(a)] *Deviations, see paragraph 7.6*

12.2 Tables: analytical results of Mefenamic Acid

Table 2 Test item Concentration check: Reference Standard solution Precision

Analytical Run	Sample Name	Concentration (µM)	Area	CV %
17 Jun 2021	RS 1	0.1	13200 10900 10900	4.4 %
	RS 2		10200 11700 10970	
05 Jul 2021	RS 3	0.1	14600 15700 15600	4.9 %
	RS 4		14300 14100 15600	

Table 3 Test Item Concentration Check: Mean Assay percentage values for test item working solutions for Range finding assay

Mefenamic Acid Solutions for Range Finding Assay – 17 June 2021						
WS ID	**WS Conc (µM)**	**Mean Assay %**	**CV %**	**WS Conc Calculated (µM)**	**Inc. Conc (µM)**	**Inc. Conc Calculated (µM)**
WS 1	625.00	99.20	6.2	620.10	500.00	496.08
WS 2	250.00	112.80	1.6	282.10	200.00	225.68
WS 3	62.50	111.00	0.8	69.40	50.00	55.52
WS 4	25.00	96.50	2	24.10	20.00	19.28
WS 5	6.25	105.10	6.8	6.60	5.00	5.28
WS 6	2.50	94.80	8.8	2.40	2.00	1.92
WS 7	0.25	87.80	5.6	0.22	0.20	0.18
WS 8	0.025	111.10	3.3	0.028	0.02	0.02

Table 4 Test Item Concentration Check: Mean Assay percentage values for test item working solutions for Main inhibition assay

Mefenamic Acid Solutions for Main Inhibition Assay - 05 Jul 2021						
WS ID	**WS Conc (µM)**	**Mean Assay %**	**CV %**	**WS Conc Calculated (µM)**	**Inc. Conc (µM)**	**Inc. Conc Calculated (µM)**
WS 1	625.00	*	*	620.10	500.0	496.08
WS 2	400.00	103.20	6.5	412.90	320.0	330.32
WS 3	250.00	*	*	282.10	200.0	225.68
WS 4	200.00	102.60	2.7	205.10	160.0	164.08
WS 5	100.00	112.60	2.1	112.60	80.0	90.08
WS 6	62.50	*	*	69.40	50.0	55.52
WS 7	25.00	*	*	24.10	20.0	19.28
WS 8	6.250	*	*	6.600	5.0	5.28

** Range finding assay working solutions that were not reinjected.*

12.3 Tables: analytical results of T3 solutions

Table 5 Substrates Concentration Check: Back-Calculated Concentrations of T3 Calibration Standards

Assay Data / Run N°	ID and Concentration (µM) of T3									
		Std 8 0.094	**Std 7 0.188**	**Std 6 0.375**	**Std 5 0.750**	**Std 4 1.500**	**Std 3 3.000**	**Std 2 6.000**	**Std 1 12.000**	**AC**
1-Jul-21 / 0	Conc µM	0.097 0.093	0.197 0.182	0.378 0.351	0.723 0.688	1.450 1.43	3.150 3.09	6.780 6.58	11.400 11.600	
	Bias%	3.0 -1.2	5.0 -3.1	1.0 6.5	-3.7 8.2	-3.5 5.6	5.0 3.0	13 10	-5.1 3.6	≤ ±15%
	n	16								≥ 12

Table 6 Substrates Concentration check: T3 Calibration Curve Parameters

Run Date	Curve Number	Slope	Intercept	R-Squared	LLOQ	ULOQ	Regression Footnote(s)
1-Jul-21	1	2.63	0.00434	0.9973	0.094	12.000	1/(x*x)

Table 7 Substrates Concentration Check: T3 Stock solution

ID – Theorical Concentration	T3 SS: Calculated Concentration mM			Assay (%)	
	Replicates	**Mean**	**CV (%)**	**Replicates**	**Mean**
T3 - 2 mM	2.30 2.31 2.27	2.29	0.91	105 106 109	114.3
T3 - 5 mM	5.29 5.38 5.39	5.35	1.03	106 108 108	107.3
T3 - 10 mM	9.96 10.8 10.6	10.45	4.20	99.6 108 106	104.5

12.4 Tables: analytical results of T4 solutions

Table 8 Substrates Concentration Check: Back-Calculated Concentrations of T4 Calibration Standards

Assay Data / Run N°		ID and Concentration (µM) of T4								
		Std 8 0.094	**Std 7 0.188**	**Std 6 0.375**	**Std 5 0.750**	**Std 4 1.500**	**Std 3 3.000**	**Std 2 6.000**	**Std 1 12.000**	**AC**
1-Jul-21 / 0	Conc µM	0.94	0.191	0.391	1.040*	1.700	3.300	5.500	9.040*	
		0.090	0.188	0.386	0.969	1.650	3.010	5.120	10.30	
	Bias%	0.0	2.0	4.0	38*	14	1.0	-8.3	-24.7*	≤ ±15%
		-4.6	0.0	3.0	8.0	10.0	0.0	-14.7	-14.1	
	n	14								≥ 12

** Did not met acceptance criteria*

Table 9 Substrates Concentration check: T4 Calibration Curve Parameters

Run Date	Curve Number	Slope	Intercept	R-Squared	LLOQ	ULOQ	Regression Footnote(s)
1-Jul-21	1	1.61	0.0648	0.9948	0.094	12.000	1/(x*x)

Table 10 Substrates Concentration Check: T4 Stock solution

ID – Theorical Concentration	T4 SS: Calculated Concentration mM			Assay (%)	
	Replicates	Mean	CV (%)	Replicates	Mean
T4 - 2 mM	2.12 2.11 2.08	2.10	0.99	106 106 104	105.3
T4 - 5 mM	4.94 4.95 4.97	4.95	0.31	98.8 98.9 99.3	99.0
T4 - 10 mM	11.3 10.9 11.3	11.17	2.07	109 104 109	107.3

12.5 Tables: analytical results of methylumbelliferone fluorescence assay

Table 11 MU fluorescence assay: Calibration Curve Parameters

Run Date	Plate Number	Slope	R-Squared	n	LLOQ	ULOQ
29-Jun-21	1	761.91	0.9992	16	1	80
30-Jun-21	1	711.40	0.9998	16	1	80
2-Jul-21	1	689.50	0.9996	16	1	80
2-Jul-21	2	640.71	0.9998	16	1	80
2-Jul-21	3	606.39	0.9998	16	1	80
6-Jul-21	1	650.37	0.9993	16	1	80
6-Jul-21	2	632.91	0.9995	16	1	80
6-Jul-21	3	628.16	0.9994	16	1	80
9-Jul-21	1	659.36	0.9989	16	1	80
9-Jul-21	2	589.86	0.9991	16	1	80
9-Jul-21	3	623.09	0.9992	16	1	80
22-Jul-21	1	622.06	0.9995	16	1	80
22-Jul-21	2	630.65	0.9988	16	1	80
22-Jul-21	3	595.97	0.9991	16	1	80
5-Aug-21	1	685.08	0.9992	16	1	80
5-Aug-21	2	630.58	0.9993	16	1	80
5-Aug-21	3	659.36	0.9989	16	1	80

Table 12 MU fluorescence assay: Precision and accuracy of MU Quality Controls

Exp/Run Date/ Run Number	QC Plates	LQC 2.5 µM				MQC 10 µM				HQC 60 µM				AC
		1	2	3	Inter-plate	1	2	3	Inter-plate	1	2	3	Inter-plate	
New HLM Batch Assay 29-Jun-21 1	Conc µM	2.81	-	-	-	9.45	-	-	-	59.04	-	-	-	
		2.94	-	-	-	9.56	-	-	-	59.64	-	-	-	
	Intra-run %Bias	12.4	-	-	-	-5.5	-	-	-	-1.6	-	-	-	≤ ± 20%
		17.6	-	-	-	-4.4	-	-	-	-0.6	-	-	-	
New HLM Batch Assay 30-Jun-21 2	Conc µM	2.66	-	-	-	8.90	-	-	-	55.88	-	-	-	
		2.75	-	-	-	9.08	-	-	-	57.16	-	-	-	
	Intra-run %Bias	6.4	-	-	-	-11.0	-	-	-	-6.9	-	-	-	≤ ± 20%
		10.0	-	-		-9.2	-	-		-4.7	-	-		
Range Finding Assay 1-Jul-21 0	Conc µM	2.61	2.56	2.59	2.57	11.18	9.35	8.59	10.06	67.02	55.46	58.34	59.56	
		2.62	2.47	2.59		11.44	9.98	9.79		61.04	58.22	57.26		
	Intra-plate %Bias	4.4	2.4	3.6	2.9	11.8	-6.5	-14.1	0.6	11.7	-7.6	-2.8	-0.7	≤ ± 20%
		4.8	-1.2	3.6		14.4	-0.2	-2.1		1.7	-3.0	-4.6		
Main Inhibition Assay 6-Jul-21 1	Conc µM	2.97	3.00	2.92	2.94	9.16	8.95	9.07	9.09	61.29	61.24	59.15	60.43	
		2.85	2.89	2.98		9.16	9.05	9.14		61.27	60.83	58.79		
	Intra-run %Bias	18.8	20.0	16.8	17.4	-8.4	-10.5	-9.3	-9.1	2.2	2.1	-1.4	0.7	≤ ± 20%
		14.0	15.6	19.2		-8.4	-9.5	-8.6		2.1	1.4	-2.0		
Main Inhibition Assay 9-Jul-21 2	Conc µM	2.83	2.92	2.99	2.91	9.10	8.22	8.41	8.63	61.28	50.87	52.71	56.57	
		2.97	2.94	2.79		9.06	8.47	8.52		64.66	54.07	55.83		
	Intra-run %Bias	13.2	16.8	19.6	16.3	-9.0	-17.8	-15.9	-13.7	2.1	-15.2	-12.2	-5.7	≤ ± 20%
		18.8	17.6	11.6		-9.4	-15.3	-14.8		7.8	-9.9	-7.0		
Main Inhibition Assay 14-Jul-21 3	Conc µM	2.83	2.90	2.97	2.94	8.88	9.12	8.91	8.89	59.20	56.62	54.45	56.35	
		2.98	2.99	2.98		8.66	9.03	8.74		58.80	56.85	52.15		
	Intra-run %Bias	13.2	16.0	18.8	17.7	-11.2	-8.8	-10.9	-11.1	-1.3	-5.6	-9.3	-6.1	≤ ± 20%
		19.2	19.6	19.2		-13.4	-9.7	-12.6		-2.0	-5.3	-13.1		

Exp/Run Date/ Run Number	QC Plates	LQC 2.5 µM 1	2	3	Inter-plate	MQC 10 µM 1	2	3	Inter-plate	HQC 60 µM 1	2	3	Inter-plate	AC
Main Inhibition Assay 22-Jul-21 4	Conc µM	2.60 2.68	2.70 2.53	2.56 2.81	2.65	9.36 9.50	9.15 9.37	9.15 9.07	9.27	61.55 60.15	61.18 62.81	60.04 59.78	60.92	
	Intra-run %Bias	4.0 7.2	8.0 1.2	2.4 12.4	5.9	-6.4 -5.0	-8.5 -6.3	-8.5 -9.3	-7.3	2.6 0.2	2.0 4.7	0.1 -0.4	1.5	≤ ± 20%
Main Inhibition Assay 5-Aug-21 5	Conc µM	2.94 2.99	2.96 2.79	2.69 2.83	2.87	8.82 9.00	8.93 8.12	8.73 8.69	8.72	61.63 62.17	57.37 54.32	58.95 62.20	59.44	
	Intra-run %Bias	17.6 19.6	18.4 11.6	7.6 13.2	14.7	-11.8 -10.0	-10.7 -18.8	-12.7 -13.1	-12.9	2.7 3.6	-4.4 -9.5	-1.8 3.7	-0.9	≤ ± 20%
Inter-run	Mean				2.81				9.11				58.88	
	S.D.				0.2				0.5				2.0	
	%CV				5.7				5.7				3.3	≤ 20%
	%Bias				12.5				-8.9				-1.9	≤ ± 20%
	n				8				8				8	≥ 6

12.6 Tables: experimental results of methylumbelliferone fluorescence assay

Table 13 MU fluorescence assay: UGTs activities of new HLM batch

Run Date / Run N°	UGT Activity (mU/mg)			
		Solvent	No Solvent	AC
29-Jun-21 / 1	r1	128.6	134.3	
	r2	93.2	121.3	≥ 80
	r3	120.1	116.0	
	Mean	114.0	123.9	≥ 80
	SD	18.5	9.4	
	CV %	16.2	7.6	≤ 20%
30-Jun-21 / 2	r1	118.5	122.2	
	r2	125.1	121.6	≥ 80
	r3	99.9	121.5	
	Mean	114.5	121.7	≥ 80
	SD	13.1	0.4	
	CV %	11.4	0.3	≤ 20%
Inter-run	Mean	114.2	122.8	≥ 80
	SD	0.4	1.5	
	CV %	0.3	1.2	≤ 20%

Table 14 MU fluorescence assay: UGTs activities in parallel with inhibition assay

Run Date / Run N°		UGT Activity (mU/mg)		
		Solvent	No Solvent	AC
1-Jul-21 / 0	Mean	116.1	111.7	≥ 80
	CV %	4.5	5.5	≤ 20%
	Bias%	1.6	-9.0	≤ 20%
6-Jul-21 / 1	Mean	97.1	101.8	≥ 80
	CV %	16.9	13.6	≤ 20%
	Bias%	-15.0	-17.1	≤ 20%
9-Jul-21 / 2	Mean	112.9	106.7	≥ 80
	CV %	18.5	16.9	≤ 20%
	Bias%	-1.2	-13.1	≤ 20%
14-Jul-21 / 3	Mean	109.2	112.7	≥ 80
	CV %	16.0	17.9	≤ 20%
	Bias%	-4.4	-8.2	≤ 20%
22-Jul-21 / 4	Mean	108.9	105.1	≥ 80
	CV %	17.8	16.6	≤ 20%
	Bias%	-4.7	-14.4	≤ 20%
5-Aug-21 / 5	Mean	123.4	123.1	≥ 80
	CV %	9.9	12.5	≤ 20%
	Bias%	8.0	0.2	≤ 20%
Inter-run	Mean	111.3	110.2	≥ 80
	CV %	7.9	6.8	≤ 20%
	Bias%	-2.6	-10.3	≤ 20%

Table 15 MU fluorescence assay: % inhibition in parallel with inhibition assay

Run Date / Run N°		% Inhibition vs Solvent		
		PC C1 500 µM	NC C1 500 µM	No Solvent
1-Jul-21 / 0	Intra-run Avg	69.2	-1.5	3.8
6-Jul-21 / 1	Intra-run Avg	74.5	3.9	-0.1
9-Jul-21 / 2	Intra-run Avg	71.9	-0.8	4.9
14-Jul-21 / 3	Intra-run Avg	74.6	0.0	-3.0
22-Jul-21 / 4	Intra-run Avg	71.1	2.2	8.3
5-Aug-21 / 5	Intra-run Avg	68.7	-1.6	0.5
AC	Intra-run Avg	>60%	<20%	<20%

12.7 Tables: analytical results of T3G

Table 16 Back-Calculated Concentrations of T3G Calibration Standards

Assay Data / Run N°	ID and Concentration (µM) T3G									
		Std 9 0.005	Std 8 0.01	Std 7 0.025	Std 6 0.05	Std 5 0.1	Std 4 0.25	Std 3 0.5	Std 2 1	Std 1 2	AC
1-Jul-21 / 0	Conc	0.0114*	0.012	0.023	0.054	0.095	0.254	0.545	1.070	2.170	
	µM	0.0047	0.017*	0.022	0.051	0.087	0.224	0.528	0.945	1.850	
	Bias%	127.0	15.0	-10.1	8.0	-4.6	2.0	9.0	7.0	9.0	≤ ±15%
		-5.6	70.0	-10.3	2.0	-12.8	-10.6	6.0	-5.5	-7.6	
	n	16									≥ 14
6-Jul-21 / 1	Conc	0.0032*	0.011	0.028	0.055	0.097	0.233	N/D*	0.953	1.910	
	µM	0.0046	0.0417*	0.025	0.046	0.103	0.221	0.546	0.987	2.090	
	Bias%	-36.2	10.0	13.0	10.0	-2.6	-6.8	N/D*	-4.7	-4.7	≤ ±15%
		-7.1	317.0	-2.0	-8.7	3.0	-11.7	9.0	-1.3	4.0	
	n	15									≥ 14
9-Jul-21 / 2	Conc	0.0067	0.009	0.027	N/D*	N/D*	0.273	0.519	1.060	2.040	
	µM	0.0050	0.0792*	0.027	0.052	0.096	0.215	0.505	0.918	1.840	
	Bias%	15.0	-6.9	9.0	N/D*	N/D*	9.0	4.0	6.0	2.0	≤ ±15%
		0.0	692.0	6.0	3.0	-4.2	-14.0	1.0	-8.2	-7.8	
	n	15									≥ 14
14-Jul-21 / 3	Conc	0.0051	0.010	0.023	0.051	0.098	0.250	0.529	0.965	1.790	
	µM	0.0050	0.010	0.023	0.052	0.115	0.271	0.663*	1.38*	1.960	
	Bias%	2.0	-3.1	-10.0	1.0	-2.3	-0.1	6.0	-3.5	-10.7	≤ ±15%
		4.0	-0.4	-9.1	4.0	15.0	8.0	33.0	38.0	-1.9	
	n	17									≥ 14
22-Jul-21 / 4	Conc	0.0057	0.009	0.021	0.046	0.108	0.225	0.563	0.934	1.750	
	µM	0.0046	0.009	0.029	0.0352*	0.100	0.250	0.569	1.150	1.910	
	Bias%	15.0	-13.0	-15.0	-7.5	8.0	-10.1	13.0	-6.6	-12.6	≤ ±15%
		-7.6	-12.0	14.0	-29.6	-0.1	0.0	14.0	15.0	-4.7	
	n	17									≥ 14
5-Aug-21 / 5	Conc	0.0047	0.0142*	0.027	0.043	0.101	0.287	0.426	1.030	1.730	
	µM	0.0052	0.010	0.0124*	0.043	0.114	0.247	0.553	0.973	2.150	
	Bias%	-5.3	42.0	9.0	-14.0	1.0	15.0	-14.9	3.0	-13.6	≤ ±15%
		5.0	2.0	-50.5	-14.1	14.0	-1.4	11.0	-2.7	8.0	
	n	16									≥ 14
Inter-Run	Mean	0.0052	0.010	0.025	0.048	0.104	0.247	0.526	0.997	1.917	
	S.D.	0.0007	0.001	0.003	0.005	0.007	0.024	0.046	0.073	0.144	
	%CV	12.9	9.2	10.7	9.3	6.9	9.8	8.8	7.3	7.5	≤ 15%
	%Bias	4.0	-3.0	1.6	-3.2	3.5	-1.1	5.3	-0.3	-4.2	≤ ±15%
	n	9	8	11	11	11	12	11	11	12	

Table 17 Calibration Curve Parameters for T3G Calibration Standards in HLM

Run Date	Run Number	Slope	Intercept	R-Squared	LLOQ	ULOQ	Regression Footnote(s)
1-Jul-21	0	1.37	0.0095	0.9955	0.005	2	1/(x*x)
6-Jul-21	1	1.41	0.0155	0.9962	0.005	2	1/(x*x)
9-Jul-21	2	1.32	0.0100	0.9969	0.005	2	1/(x*x)
9-Jul-21	3	0.78	0.0075	0.9967	0.005	2	1/(x*x)
22-Jul-21	4	0.93	0.0076	0.9922	0.005	2	1/(x*x)
5-Aug-21	5	1.14	0.0146	0.9936	0.005	2	1/(x*x)

Table 18 Precision and accuracy of T3G Quality Control in the five validated runs

Run Date	Run Number	QC Conc. µM	LQC 0.015	MQC 0.1	HQC 1.5	AC
1-Jul-21	0	Conc µM	0.0145 0.0131*	0.114 0.101	1.40 1.38	
		Intra-run %Bias	-9.7 -18.1	14.0 1.0	-3.9 -5.0	≤ ± 15%
		n		5		≥ 4
6-Jul-21	1	Conc µM	0.0162 0.0103*	0.113 0.114	1.60 1.43	
		Intra-run %Bias	1.0 -35.5*	13.0 14.0	7.0 -4.9	≤ ± 15%
		n		6		≥ 4
9-Jul-21	2	Conc µM	0.0181 0.0142	N/D* 0.092	1.48 1.28	
		Intra-run %Bias	13.0 -11.0	N/D* -7.5	-1.6 -14.7	≤ ± 15%
		n		5		≥ 4
14-Jul-21	3	Conc µM	0.0138 N/D*	0.114 0.114	1.45 1.41	
		Intra-run %Bias	-13.5 N/D*	14.0 14.0	-3.2 -6.2	≤ ± 15%
		n		5		≥ 4
22-Jul-21	4	Conc µM	0.0175 N/D*	0.110 0.111	1.65 N/D	
		Intra-run %Bias	9.0 N/D*	10.0 11.0	10.0 N/D*	≤ ± 15%
		n		4		≥ 4
5-Aug-21	5	Conc µM	0.0355* 0.0177	0.088 0.093	1.72 1.44	
		Intra-run %Bias	256.0* 14.0	12.0 10.0	-13.5 -12.0	≤ ± 15%
		n		5		≥ 4
Inter-run		Mean S.D. %CV %Bias n	0.0163 0.0019 11.4 8.3 7	0.1055 0.0110 10.4 5.5 9	1.4956 0.1363 9.1 -0.3 10	 ≤ 15% ≤ ± 15%

** Did not met acceptance criteria*

Table 19 Selectivity Effect Test of T3G in HLM in the five valid runs

Run Date	Run Number	Analyte Peak Area		
		T3G	T3	Fexofenadine
6-Jul-21	1	No Peak N/D No Peak	3.98E+06 N/D 3.61E+06	1.44E+05 N/D 1.62E+05
9-Jul-21	2	No Peak N/D No Peak	3.11E+06 N/D 2.33E+06	1.62E+05 N/D 1.43E+05
14-Jul-21	3	No Peak N/D No Peak	2.60E+06 N/D 2.51E+06	1.77E+05 N/D 1.40E+05
22-Jul-21	4	No Peak N/D No Peak	2.66E+06 N/D 2.26E+06	1.72E+05 N/D 1.53E+05
5-Aug-21	5	No Peak N/D No Peak	2.54E+06 N/D 2.56E+06	1.20E+05 N/D 1.83E+05

Table 20 Selectivity Effect Test in HLM in T3G formation w/o UDPGA incubation

Run Date	Run Number	Analyte Peak Area					
		T3 5 µM		T3 5 µM		T3 10 µM	
		T3G	T3	T3G	T3	T3G	T3
1-Jul-21	1	No Peak	3.30E+06	No Peak	3.19E+06	No Peak	4.90E+06
		No Peak	3.36E+06	No Peak	3.29E+06	No Peak	5.64E+06
		No Peak	3.50E+06	No Peak	2.70E+06	No Peak	5.43E+06
6-Jul-21	1	No Peak	1.20E+06	No Peak	3.40E+06	No Peak	4.86E+06
		No Peak	1.37E+06	No Peak	3.35E+06	No Peak	5.47E+06
		No Peak	6.40E+05	No Peak	3.22E+06	No Peak	5.56E+06
9-Jul-21	2	No Peak	9.71E+05	No Peak	3.30E+06	No Peak	5.55E+06
		No Peak	1.39E+06	No Peak	3.37E+06	No Peak	5.32E+06
		No Peak	1.48E+06	No Peak	3.19E+06	No Peak	5.81E+06
14-Jul-21	3	No Peak	3.90E+05	No Peak	2.38E+06	No Peak	3.21E+06
		No Peak	9.81E+05	No Peak	2.53E+06	No Peak	4.25E+06
		No Peak	9.45E+05	No Peak	2.46E+06	No Peak	4.78E+06
22-Jul-21	4	No Peak	1.05E+06	No Peak	2.72E+06	No Peak	4.38E+06
		No Peak	1.04E+06	No Peak	2.61E+06	No Peak	4.25E+06
		No Peak	1.12E+06	No Peak	2.97E+06	No Peak	4.74E+06
5-Aug-21	5	No Peak	1.26E+06	No Peak	3.17E+06	No Peak	5.02E+06
		No Peak	1.27E+06	No Peak	3.10E+06	No Peak	5.06E+06
		No Peak	1.44E+06	No Peak	2.92E+06	No Peak	5.54E+06

12.8 Tables: experimental results of T3G formation/inhibition

Table 21 Range finding Assay: effect of Mefenamic Acid and No Solvent on the T3G formation

Range Finding Assay										
1-Jul-21		**TI**	**TI**	**TI**	**TI**	**TI**	**TI**	**TI**	**TI**	**NC**
ID		**C1**	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**	**C1**
Conc µM		**500.0**	**200.0**	**50.0**	**20.0**	**5.0**	**2.5**	**0.2**	**0.02**	**500.0**
T3 5 µM										
% activities vs Solvent	r1	8.8	35.2	67.0	97.0	93.8	95.7	104.0	114.9	113.6
	r2	8.8	28.9	74.7	92.6	97.7	79.8	76.0	118.1	100.2
	r3	8.4	30.4	72.8	90.0	98.9	93.2	113.6	113.0	108.5
	Avg	8.7	31.5	71.5	93.2	96.8	89.6	97.9	115.3	107.4
	CV %	3.0	10.6	5.6	3.8	2.7	9.6	20.0	2.2	6.3
% Inhibition	Avg	**91.3**	**68.5**	**28.5**	**6.8**	**3.2**	**10.4**	**2.1**	**-15.3**	**-7.4**
T3 5 µM										
% activities vs Solvent	r1	8.6	28.3	82.0	94.7	97.7	97.7	109.0	114.3	108.3
	r2	7.6	34.4	78.9	106.0	109.8	98.5	113.5	110.5	105.3
	r3	5.3	34.4	56.8	85.0	103.8	114.3	105.3	109.0	90.2
	Avg	7.2	32.4	72.6	95.2	103.8	103.5	109.3	111.3	101.3
	CV %	23.7	10.8	18.9	11.1	5.8	9.0	3.8	2.4	9.5
% Inhibition	Avg	**92.8**	**67.6**	**27.4**	**4.8**	**-3.8**	**-3.5**	**-9.3**	**-11.3**	**-1.3**
T3 10 µM										
% activities vs Solvent	r1	12.9	40.6	81.1	100.1	152.9	95.7	112.6	108.6	101.0
	r2	14.5	35.9	76.7	97.9	141.4	98.8	103.2	93.1	105.5
	r3	15.2	36.9	76.7	105.5	139.6	93.5	105.5	105.9	98.8
	Avg	14.2	37.8	78.1	101.2	144.6	96.0	107.1	102.5	101.8
	CV %	8.1	6.6	3.3	3.8	5.0	2.8	4.5	8.1	3.3
% Inhibition	Avg	**85.8**	**62.2**	**21.9**	**-1.2**	**-44.6**	**4.0**	**-7.1**	**-2.5**	**-1.8**

Table 22 Main Inhibition Assay: T3G formation in the "Solvent" in the five valid runs

Run Date / Run N°	T3G Concentrations µM			T3G Formation Rate			AC
	T3	2 µM	5 µM	10 µM	2 µM	5 µM	10 µM	
6-Jul-21 / 1	r1	0.0493	0.132	0.251	0.274	0.733	1.394	
	r2	0.0355	0.135	0.217	0.197	0.750	1.206	≥ 0.400 [(a)]
	r3	0.0468	0.148	0.227	0.260	0.822	1.261	
	Mean	0.0439	0.138	0.232	0.244	0.769	1.287	≥ 0.400 [(a)]
	SD	0.0074	0.009	0.017	0.041	0.047	0.097	
	CV %	16.8	6.1	7.5	16.8	6.1	7.5	≤20%
9-Jul-21 / 2	r1	0.0570	0.138	0.185	0.317	0.767	1.028	
	r2	0.0422	0.150	0.177	0.234	0.833	0.983	≥ 0.400 [(a)]
	r3	0.0542	0.138	0.182	0.301	0.767	1.011	
	Mean	0.0511	0.142	0.181	0.284	0.789	1.007	≥ 0.400 [(a)]
	SD	0.0079	0.007	0.004	0.044	0.038	0.022	
	CV %	15.4	4.9	2.2	15.4	4.9	2.2	≤20%
14-Jul-21 / 3	r1	0.0616	0.167	0.237	0.342	0.928	1.317	
	r2	0.0458	0.201	0.2	0.254	1.117	1.111	≥ 0.400 [(a)]
	r3	0.0625	0.176	0.209	0.347	0.978	1.161	
	Mean	0.0566	0.181	0.215	0.315	1.007	1.196	≥ 0.400 [(a)]
	SD	0.0094	0.018	0.019	0.052	0.098	0.107	
	CV %	16.6	9.7	9.0	16.6	9.7	9.0	≤20%
22-Jul-21 / 4	r1	0.0143	0.0454	0.0825	0.079	0.252	0.458	
	r2	0.0101	0.0483	0.0742	0.056	0.268	0.412	≥ 0.400 [(a)]
	r3	0.0106	0.0434	0.0719	0.059	0.241	0.399*	
	Mean	0.0117*	0.046*	0.076*	0.065*	0.254*	0.423*	≥ 0.400 [(a)]
	SD	0.0023	0.002	0.006	0.013	0.014	0.031	
	CV %	19.7	5.4	7.3	19.7	5.4	7.3	≤20%
5-Aug-21 / 5	r1	0.055	0.154	0.186	0.306	0.856	1.033	
	r2	0.0658	0.133	0.171	0.366	0.739	0.950	≥ 0.400 [(a)]
	r3	0.0602	0.15	0.175	0.334	0.833	0.972	
	Mean	0.0603	0.146	0.177	0.335	0.809	0.985	≥ 0.400 [(a)]
	SD	0.0054	0.011	0.008	0.030	0.062	0.043	
	CV %	9.0	7.7	4.4	9.0	7.7	4.4	≤20%
Inter-run	Mean	0.0530	0.121	0.201	0.2944	0.844	1.119	≥ 0.400 [(a)]
	SD	0.0072	0.020	0.026	0.0398	0.111	0.147	
	CV %	13.5	16.4	13.1	13.5	13.1	13.1	≤20%

[(a)] *Acceptance Criteria: ≥ 0.400 pmol/mg protein/min of T3G when T3 was incubated at 10 µM*
** Did not met acceptance criteria*

Table 23 Main Inhibition Assay: T3G formation in the "No Solvent" in the five valid runs

Run Date/ Run N°	T3G Concentrations µM				T3G % activity vs solvent			AC
	T3	2 µM	5 µM	10 µM	2 µM	5 µM	10 µM	
6-Jul-21 / 1	r1	0.0467	0.136	0.246	106.5	98.3	106.2	
	r2	0.0451	0.138	0.244	102.8	99.8	105.3	80-120% [a]
	r3	0.0456	0.126	0.218	104.0	91.1	94.1	
	Mean	0.0458	0.133	0.236	104.4	96.4	101.9	80-120% [a]
	SD	0.0008	0.006	0.016	1.9	4.6	6.7	
	CV %	1.8	4.8	6.6	1.8	4.8	6.6	≤20%
9-Jul-21 / 2	r1	0.0542	0.136	0.161	106.0	95.8	88.8	
	r2	0.0524	0.146	0.188	102.5	102.8	103.7	80-120% [a]
	r3	0.053	0.135	0.162	103.7	95.1	89.3	
	Mean	0.0532	0.139	0.170	104.0	97.9	93.9	80-120% [a]
	SD	0.0009	0.006	0.015	1.8	4.3	8.4	
	CV %	1.7	4.4	9.0	1.7	4.4	9.0	≤20%
14-Jul-21 / 3	r1	0.0627	0.213	0.185	110.7	117.5	85.9	
	r2	0.0759	0.168	0.173	134.0	92.6	80.3	80-120% [a]
	r3	0.0547	0.154	0.168	96.6	84.9	78.0	
	Mean	0.0644	0.178	0.175	113.8	98.3	81.4	80-120% [a]
	SD	0.0107	0.031	0.009	18.9	17.0	4.1	
	CV %	16.6	17.3	5.0	16.6	17.3	5.0	≤20%
22-Jul-21 / 4	r1	0.0344	0.133	0.192	88.4[b]	113.4[b]	110.3[b]	
	r2	0.0356	0.116	0.161	91.4[b]	98.9[b]	92.5[b]	80-120% [a]
	r3	0.0468	0.103	0.169	120.2[b]	87.8[b]	97.1[b]	
	Mean	0.0389	0.117	0.174	100.0[b]	100.0[b]	100.0[b]	80-120% [a]
	SD	0.0068	0.015	0.016	17.6	12.8	9.2	
	CV %	17.6	12.8	9.2	17.6	12.8	9.2	≤20%
5-Aug-21 / 5	r1	0.0495	0.126	0.243	82.0	86.5	137.0	
	r2	0.0455	0.15	0.186	75.4	103.0	104.9	80-120% [a]
	r3	0.049	0.103	0.174	81.2	70.7	98.1	
	Mean	0.0480	0.126	0.201	79.6	86.7	113.3	80-120% [a]
	SD	0.0022	0.024	0.037	3.6	16.1	20.8	
	CV %	4.5	18.6	18.3	4.5	18.6	18.3	≤20%
Inter-run	Mean	0.0501	0.139	0.191	100.4	94.8	97.6	80-120%
	SD	0.0095	0.024	0.028	14.6	5.5	13.4	
	CV %	19.0	16.9	14.5	14.6	5.8	13.8	≤20%

[a] *Acceptance Criteria: the percentage activity versus solvent were 80 – 120 %*
(b) % Activity values were calculated versus mean of No solvent

Table 24 Main Inhibition Assay: T3G formation in the "Negative Control" in the five valid runs

Run Date/ Run N°	T3G Concentrations µM				T3G % activity vs solvent			AC
	T3	2 µM	5 µM	10 µM	2 µM	5 µM	10 µM	
6-Jul-21 / 1	r1	0.0464	0.134	0.25	105.8	96.9	107.9	
	r2	0.0537	0.12	0.251	122.4	86.7	108.3	80-120% [a]
	r3	0.0542	0.127	0.242	123.6	91.8	104.5	
	Mean	0.0514	0.127	0.248	117.2	91.8	106.9	80-120% [a]
	SD	0.0044	0.007	0.005	10.0	5.1	2.1	
	CV %	8.5	5.5	2.0	8.5	5.5	2.0	≤20%
9-Jul-21 / 2	r1	0.0409	0.117	0.182	80.0	82.4	100.4	
	r2	0.0499	0.126	0.208	97.6	88.7	114.7	80-120% [a]
	r3	0.039	0.128	0.185	76.3	90.1	102.0	
	Mean	0.0433	0.124	0.192	84.6	87.1	105.7	80-120% [a]
	SD	0.0058	0.006	0.014	11.4	4.1	7.8	
	CV %	13.5	4.7	7.4	13.5	4.7	7.4	≤20%
14-Jul-21 / 3	r1	N/D*	0.158	0.193	N/D*	87.1	89.6	
	r2	0.0427	0.154	0.247	75.4	84.9	114.7	80-120% [a]
	r3	0.0539	0.134	0.239	95.2	73.9	111.0	
	Mean	0.0483	0.149	0.226	85.3	82.0	105.1	80-120% [a]
	SD	0.0079	0.013	0.029	14.0	7.1	13.5	
	CV %	16.4	8.6	12.9	16.4	8.6	12.9	≤20%
22-Jul-21 / 4	r1	0.0414	0.14	0.203	106.3[b]	119.3[b]	116.7[b]	
	r2	0.0396	0.151	0.184	101.7[b]	128.7[b]	105.7[b]	80-120% [a]
	r3	0.0458	0.119	0.188	117.6[b]	101.4[b]	108.0[b]	
	Mean	0.0423	0.137	0.192	108.6[b]	116.5[b]	110.2[b]	80-120% [a]
	SD	0.0032	0.016	0.010	8.2	13.9	5.8	
	CV %	7.5	11.9	5.2	7.5	11.9	5.2	≤20%
5-Aug-21 / 5	r1	0.0485	0.141	0.2	80.4	96.8	112.8	
	r2	0.0447	0.13	0.176	74.1	89.2	99.2	80-120% [a]
	r3	0.0478	0.113	0.173	79.2	77.6	97.6	
	Mean	0.0470	0.128	0.183	77.9	87.9	103.2	80-120% [a]
	SD	0.0020	0.014	0.015	3.4	9.7	8.3	
	CV %	4.3	11.0	8.1	4.3	11.0	8.1	≤20%
Inter-run	Mean	0.0465	0.133	0.208	94.7	93.0	106.2	80-120%
	SD	0.0037	0.010	0.028	17.1	13.6	2.6	
	CV %	8.1	7.6	13.3	18.1	14.6	2.4	≤20%

[a] *Acceptance Criteria: the percentage activity versus solvent were 80 – 120%*
[b] *% Activity values were calculated versus mean of No solvent*

Table 25 Main Inhibition Assay: T3G formation in the Test Item in the five valid runs (T3 2 µM)

Run Date/ Run N°	T3 2 µM % Activity vs Solvent									AC
	Mefenamic Acid	C1 500.0	C2 320.0	C3 200.0	C4 160.0	C5 80.0	C6 50.0	C7 20.0	C8 5.0	
6-Jul-21 / 1	r1	LLOQ	5.3	16.6	27.6	44.0	46.3	82.1	98.9	
	r2	LLOQ	5.8	13.7	32.8	42.6	49.5	73.2	91.9	
	r3	LLOQ	4.6	20.5	27.6	48.8	53.8	70.4	107.6	
	Mean	LLOQ	5.2	17.0	29.3	45.1	49.8	75.2	99.5	
	SD	-	0.6	3.4	3.0	3.2	3.8	6.1	7.9	
	CV %	-	11.1	19.9	10.3	7.2	7.6	8.1	7.9	≤20%
9-Jul-21 / 2	r1	LLOQ	11.0	22.7	31.1	51.6	51.0	77.2	87.4	
	r2	LLOQ	11.1	18.6	29.1	44.2	52.6	85.1	86.8	
	r3	LLOQ	12.6	23.9	23.1	55.9	52.0	82.3	83.1	
	Mean	LLOQ	11.6	21.7	27.8	50.6	51.9	81.6	85.8	
	SD	-	0.9	2.8	4.2	5.9	0.8	4.0	2.3	
	CV %	-	7.9	12.8	15.1	11.7	1.5	4.9	2.7	≤20%
14-Jul-21 / 3	r1	LLOQ	LLOQ*	2.8*	N/D*	15.9*	50.1	58.4	68.5	
	r2	LLOQ	3.2	14.7	26.0	34.8	65.9	81.9	91.1	
	r3	LLOQ	3.0	13.4	25.6	44.5	47.9	72.0	93.6	
	Mean	LLOQ	3.1	14.1	25.8	39.6	54.6	70.8	84.4	
	SD	-	0.2	0.9	0.2	6.9	9.8	11.8	13.8	
	CV %	-	6.0	6.6	1.0	17.3	17.9	16.7	16.4	≤20%
22-Jul-21 / 4[(b)]	r1	LLOQ	LLOQ*	20.5	33.6	51.6	47.3	80.7	104.0	
	r2	LLOQ	LLOQ*	22.7	41.9	50.1	36.5	86.6	100.2	
	r3	LLOQ	5.1	21.4	46.2	54.7	48.8	80.4	104.0	
	Mean	LLOQ	5.1	21.5	40.6	52.1	44.2*	82.5	102.7	
	SD	-	-	1.1	6.4	2.4	6.7	3.5	2.2	
	CV %	-	-	5.2	15.7	4.5	15.2	4.2	2.2	≤20%
5-Aug-21 / 5	r1	LLOQ	2.5	14.7	33.3	51.4	37.3	72.8	76.1	
	r2	LLOQ	LLOQ*	14.4	36.3	25.2*	43.6	68.8	75.7	
	r3	LLOQ	2.1	18.4	31.0	46.4	46.6	75.2	86.4	
	Mean	LLOQ	2.3	15.8	33.5	48.9	42.5	72.3	79.4	
	SD	-	0.3	2.2	2.7	3.5	4.7	3.3	6.0	
	CV %	-	12.1	14.1	7.9	7.2	11.2	4.5	7.6	≤20%
Inter-run	Mean	LLOQ	5.5	18.0	31.4	47.3	48.6	76.5	90.4	
	SD	-	3.6	3.4	5.9	5.0	5.1	5.3	10.2	
	CV %	-	66.5	19.1	18.7	10.6	10.6	7.0	11.2	≤20%

[(b)] *% Activity values were calculated versus mean of No solvent*
LLOQ = T3G concentration was lower than 0.005%. so the % activity could not calculate
** Did not met acceptance criteria.*

Table 26 Main Inhibition Assay: T3G formation in the Test Item in the five validated runs (T3 5 µM)

Run Date/ Run N°	T3 5 µM % Activity vs Solvent								AC
	Mefenamic Acid	C1 500.0	C2 320.0	C3 200.0	C4 160.0	C5 80.0	C6 50.0	C7 20.0	C8 5.0	
6-Jul-21 / 1	r1	4.9	13.4	24.4	37.3	53.6	59.2	81.0	99.0	
	r2	5.5	14.3	30.4	28.1	51.5	53.6	76.6	95.4	
	r3	4.4	14.2	28.2	33.1	47.3	40.3	75.2	86.0	
	Mean	4.9	14.0	27.6	32.8	50.8	51.0	77.6	93.5	
	SD	0.6	0.5	3.0	4.6	3.2	9.7	3.0	6.7	
	CV %	11.5	3.3	11.0	14.0	6.4	19.1	3.9	7.2	≤20%
9-Jul-21 / 2	r1	6.5	17.7	24.6	40.1	54.7	68.6	95.1	85.9	
	r2	5.7	16.1	26.2	37.4	53.5	59.8	65.3	90.1	
	r3	6.3	15.4	25.6	34.3	53.4	56.1	81.7	87.3	
	Mean	6.2	16.4	25.5	37.3	53.8	61.5	80.7	87.8	
	SD	0.4	1.2	0.8	2.9	0.8	6.4	14.9	2.2	
	CV %	6.9	7.3	3.2	7.8	1.4	10.4	18.5	2.5	≤20%
14-Jul-21 / 3	r1	3.6	14.2	27.7	29.3	51.6	53.6	80.0	90.4	
	r2	4.1	14.0	21.5	36.0	43.4	51.7	96.0	80.0	
	r3	LLOQ*	11.7	25.9	26.0	34.4	60.7	67.3	81.1	
	Mean	3.9	13.3	25.0	30.4	43.1	55.3	81.1	83.8	
	SD	0.4	1.4	3.2	5.1	8.6	4.7	14.4	5.8	
	CV %	10.2	10.4	12.9	16.7	19.9	8.6	17.7	6.9	≤20%
22-Jul-21 / 4[(b)]	r1	4.4	6.6	25.5	44.7	66.6	57.5	91.2	102.3	
	r2	3.6	5.9	33.1	36.7	72.7	59.7	119.3	108.2	
	r3	3.1	5.7	22.8	35.9	53.9	51.6	84.9	82.8	
	Mean	3.7	6.1*	27.1	39.1	64.4	56.3	98.5	97.8	
	SD	0.7	0.5	5.3	4.8	9.6	4.1	18.3	13.3	
	CV %	18.1	8.3	19.6	12.4	14.8	7.4	18.6	13.6	≤20%
5-Aug-21 / 5	r1	4.8	14.1	24.8	36.5	48.2	64.6	85.1	85.1	
	r2	5.5	12.6	24.7	38.0	52.9	61.9	73.5	102.3	
	r3	4.6	9.6	20.8	38.4	43.4	44.7	63.7	76.9	
	Mean	5.0	12.1	23.4	37.6	48.1	57.1	74.1	88.1	
	SD	0.5	2.3	2.3	1.1	4.7	10.8	10.7	13.0	
	CV %	9.2	19.0	9.7	2.8	9.8	18.9	14.5	14.7	≤20%
Inter-run	Mean	4.7	12.4	25.7	35.5	52.1	56.2	82.4	90.2	
	SD	1.0	3.8	1.7	3.6	7.9	3.8	9.4	5.5	
	CV %	21.1	31.1	6.6	10.3	15.3	6.7	11.4	6.1	≤20%

[(b)] *% Activity values were calculated versus mean of No solvent*

LLOQ = T3G concentration was lower than 0.005%. so the % activity could not calculate

** Did not met acceptance criteria*

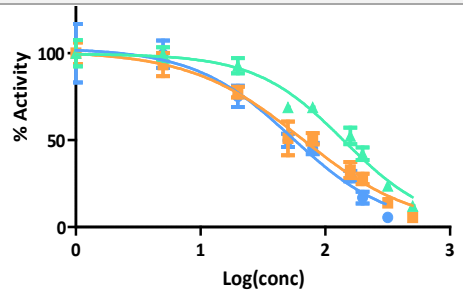
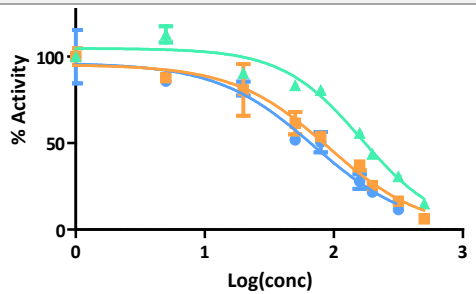
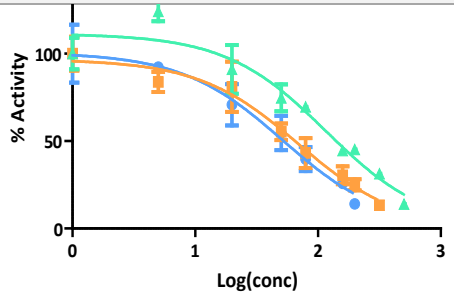
Table 27 Main Inhibition Assay: T3G formation in the Test Item in the five validated runs (T3 10 µM)

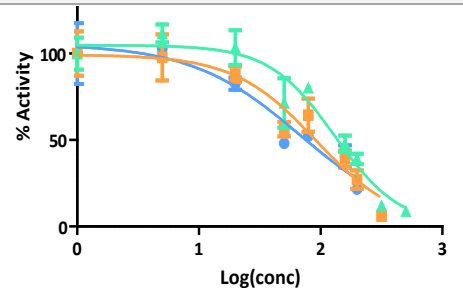
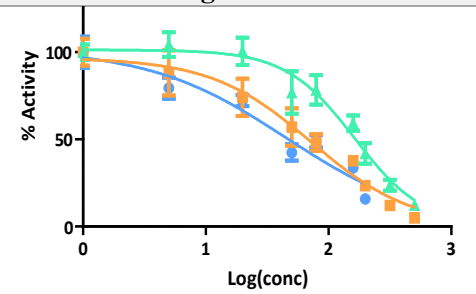
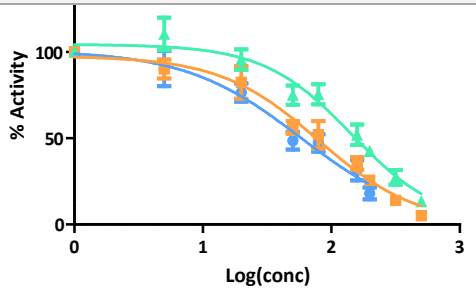
Run Date/ Run N°	T3 10 µM % Activity vs Solvent								AC
	Mefenamic Acid	C1 500.0	C2 320.0	C3 200.0	C4 160.0	C5 80.0	C6 50.0	C7 20.0	C8 5.0	
6-Jul-21 / 1	r1	11.8	24.2	45.8	52.7	70.8	71.2	97.6	103.6	
	r2	12.0	24.8	42.2	57.0	68.2	67.8	88.9	98.8	
	r3	12.2	22.0	38.5	47.5	67.3	67.3	91.9	95.4	
	Mean	12.0	23.7	42.1	52.4	68.8	68.8	92.8	99.3	
	SD	0.2	1.5	3.6	4.8	1.8	2.1	4.4	4.1	
	CV %	1.5	6.2	8.7	9.1	2.6	3.1	4.7	4.1	≤20%
9-Jul-21 / 2	r1	14.3	28.5	45.2	56.3	82.2	85.5	90.4	115.8	
	r2	15.4	30.8	43.4	56.3	79.4	81.1	N/D*	115.3	
	r3	15.2	32.9	42.5	54.9	80.0	83.3	N/D*	107.5	
	Mean	15.0	30.7	43.7	55.8	80.5	83.3	90.4	112.9	
	SD	0.6	2.2	1.4	0.8	1.5	2.2	-	4.6	
	CV %	3.7	7.1	3.1	1.4	1.8	2.6	-	4.1	≤20%
14-Jul-21 / 3	r1	12.3	29.3	45.2	44.0	70.1	78.9	75.2	125.4	
	r2	13.9	33.2	45.9	42.1	71.5	79.4	96.1	129.1	
	r3	15.7	31.3	44.3	47.4	66.9	65.9	101.7	118.0	
	Mean	13.9	31.2	45.1	44.5	69.5	74.8	91.0	124.1	
	SD	1.7	2.0	0.8	2.7	2.4	7.6	14.0	5.7	
	CV %	12.3	6.2	1.9	6.0	3.4	10.2	15.3	4.6	≤20%
22-Jul-21 / 4[(b)]	r1	8.6	10.8	42.0	53.0	78.7	87.9	114.9	114.9	
	r2	9.0	12.4	39.3	46.8	83.3	62.6	98.3	114.4	
	r3	8.6	13.0	36.0	43.7	78.7	63.8	97.1	105.7	
	Mean	8.7*	12.0*	39.1	47.9	80.3	71.5	103.4	111.7	
	SD	0.2	1.1	3.0	4.7	2.7	14.3	10.0	5.2	
	CV %	2.5	9.3	7.6	9.9	3.3	20.0	9.6	4.6	≤20%
5-Aug-21 / 5	r1	11.0	26.5	46.3	55.0	88.0	68.2	107.1	111.7	
	r2	11.6	24.1	44.3	59.2	74.4	71.6	102.6	97.6	
	r3	14.2	20.3	35.2	63.7	72.7	90.8	91.9	103.8	
	Mean	12.3	23.6	41.9	59.3	78.4	76.9	100.6	104.3	
	SD	1.7	3.1	5.9	4.4	8.3	12.2	7.8	7.1	
	CV %	13.9	13.2	14.1	7.4	10.6	15.8	7.8	6.8	≤20%
Inter-run	Mean	12.4	24.3	42.4	52.0	75.5	75.0	95.7	110.5	
	SD	2.4	7.7	2.3	5.9	5.9	5.6	5.9	9.5	
	CV %	19.3	31.9	5.3	11.4	7.8	7.4	6.2	8.6	≤20%

[(b)] *% Activity values were calculated versus mean of No solvent*

** Did not met acceptance criteria*

Table 28 Main Inhibition Assay: IC50 Curve of T3G in presence of Test Item in the five valid runs

6-Jul-21 / RUN 1					**9-Jul-21 / RUN 2**					**14-Jul-21 / RUN 3**				
														
T3G	**2 µM**	**5 µM**	**10 µM**	**AC**	**T3G**	**2 µM**	**5 µM**	**10 µM**	**AC**	**T3G**	**2 µM**	**5 µM**	**10 µM**	**AC**
IC50	53.92	65.17	140.3		IC50	70.46	90.52	163.6		IC50	51.82*	69.33	116.0	
R-square	0.9524	0.9676	0.9683	≥ 0.9000	R-square	0.9533	0.9652	0.9754	≥ 0.9000	R-square	0.9236	0.9412	0.9255	≥ 0.9000
n	23	25	27	≥ 20	n	24	27	25	≥ 20	n	17*	24	27	≥ 20

22-Jul-21 / RUN 4					**5-Aug-21 / RUN 5**					**Inter-Run**				
														
T3G	**2 µM**	**5 µM**	**10 µM**	**AC**	**T3G**	**2 µM**	**5 µM**	**10 µM**	**AC**	**T3G**	**2 µM**	**5 µM**	**10 µM**	**AC**
IC50	68.02	91.87	130.5		IC50	45.09	72.49	162.9		IC50	57.42	79.52	144.2	
R-square	0.9156	0.9112	0.9371	≥ 0.9000	R-square	0.9228	0.9459	0.9496	≥ 0.9000	R-square	0.9513	0.9671	0.9559	≥ 0.9000
n	23	23	27	≥ 20	n	20	27	27	≥ 20	n	35	43	43	≥ 33

** Did not met acceptance criteria*

Table 29 Main Inhibition Assay: Summary inhibition on the T3G formation

T3G % Inhibition vs Solvent									
ID	**TI C1**	**TI C2**	**TI C3**	**TI C4**	**TI C5**	**TI C6**	**TI C7**	**TI C8**	**NC C1**
Conc µM	**500.0**	**320.0**	**200.0**	**160.0**	**80.0**	**50.0**	**20.0**	**5.0**	**500.0**
T3 2 µM									
Run 1	LLOQ	LLOQ	83.0	70.7	54.9	50.2	24.8	0.5	-17.2
Run 2	LLOQ	88.4	78.3	72.2	49.4	48.1	18.4	14.2	15.4
Run 3	LLOQ	85.9	85.9	74.2	60.4	45.4	29.2	15.6	14.7
Run 4	LLOQ	LLOQ	78.5	59.4	47.9	55.8	17.5	-2.7	-8.6
Run 5	LLOQ	LLOQ	84.2	66.5	51.1	57.5	27.7	20.6	22.1
Inter Run	**LLOQ**	**87.1**	**82.0**	**68.6**	**52.7**	**51.4**	**23.5**	**9.6**	**5.3**
T3 5 µM									
Run 1	95.1	86.0	72.4	67.2	49.2	49.0	22.4	6.5	8.2
Run 2	93.8	83.6	74.5	62.7	46.2	38.5	19.3	12.2	12.9
Run 3	96.1	86.7	75.0	69.6	56.9	44.7	18.9	16.2	18.0
Run 4	LLOQ	93.9	72.9	60.9	35.6	43.7	1.5	2.2	-16.5
Run 5	95.0	87.9	76.6	62.4	51.9	42.9	25.9	11.9	12.1
Inter Run	**95.0**	**87.6**	**74.3**	**64.5**	**47.9**	**43.8**	**17.6**	**9.8**	**7.0**
T3 10 µM									
Run 1	88.0	76.3	57.9	47.6	31.2	31.2	7.2	0.7	-6.9
Run 2	85.0	69.3	56.3	44.2	19.5	16.7	9.6	-12.9	-5.7
Run 3	86.1	68.8	54.9	55.5	30.5	25.2	9.0	-24.1	-5.1
Run 4	91.3	88.0	60.9	52.1	19.7	28.5	-3.4	-11.7	-10.2
Run 5	87.7	76.4	58.1	40.7	21.6	23.1	-0.6	-4.3	-3.2
Inter Run	**87.6**	**75.7**	**57.6**	**48.0**	**24.5**	**25.0**	**4.3**	**-10.5**	**-6.2**

LLOQ = T3G concentration was lower than 0.005%. so the % inhibition could not calculate

Table 30 Main Inhibition Assay: summary T3G IC50 curve

IC50 T3G	T3 2 µM	T3 5 µM	T3 10 µM
Run 1	53.92	65.17	140.30
Run 2	70.46	90.52	163.60
Run 3	51.82	69.33	116.00
Run 4	68.02	91.87	130.50
Run 5	45.09	72.49	162.90
Avg	**57.86**	**77.88**	**142.66**
CV%	**18.87**	**15.98**	**14.50**
Inter-Run	57.42	79.52	144.1

12.9 Tables: analytical results of T4G

Table 31 Back-Calculated Concentrations of T4G Calibration Standards

Assay Data / Run N°	T4G ID and Concentration (µM)									
		Std 9 0.005	**Std 8 0.01**	**Std 7 0.025**	**Std 6 0.05**	**Std 5 0.1**	**Std 4 0.25**	**Std 3 0.5**	**Std 2 1**	**Std 1 2**	**AC**
1-Jul-21 / 0	Conc	0.0050	0.0071*	0.023	0.051	0.101	0.263	0.572	0.946	2.020	
	µM	0.0052	0.010	0.023	0.053	0.093	0.248	0.533	0.882	2.060	
	Bias%	1.0	-29.2	-6.6	1.0	1.0	5.0	14.0	-5.4	1.0	≤ ±15%
		4.0	-4.9	-6.6	6.0	-7.3	-0.7	7.0	-11.8	3.0	
	n	16									≥ 14
6-Jul-21 / 1	Conc	N/D*	0.011	0.026	0.049	0.098	0.259	0.505	0.954	1.920	
	µM	0.0045	0.0120*	0.027	0.056	0.091	0.241	0.541	0.892	1.890	
	Bias%	N/D*	14.0	5.0	-2.1	-2.4	3.0	1.0	-4.6	-3.8	≤ ±15%
		-9.8	20.0	7.0	13.0	-9.1	-3.5	8.0	-10.8	-5.7	
	n	15									≥ 14
9-Jul-21 / 2	Conc	0.334*	0.010	0.027	0.058	0.094	0.247	0.514	0.921	1.990	
	µM	0.0047	0.011	0.029	0.049	0.098	0.214	N/D*	0.939	2.070	
	Bias%	6590.0	-3.8	6.0	15.0	-6.0	-1.3	3.0	-7.9	-0.4	≤ ±15%
		-6.8	8.0	15.0	-1.9	-2.4	-14.4	N/D*	-6.1	4.0	
	n	15									≥ 14
14-Jul-21 / 3	Conc	0.0051	0.009	0.019	0.046	0.090	0.288	0.475	1.030	2.020	
	µM	N/D*	0.010	0.026	0.050	0.086	0.237	0.569	1.110	1.930	
	Bias%	2.0	-5.5	-23.7	-7.2	-10.5	15.0	-5.1	3.0	1.0	≤ ±15%
		N/D	4.0	2.0	-0.5	-14.5	-5.1	14.0	11.0	-3.6	
	n	17									≥ 14
22-Jul-21 / 4	Conc	0.0052	0.010	0.027	0.050	0.088	0.233	0.529	1.070	2.050	
	µM	0.0049	0.0122*	0.026	0.047	0.088	0.218	0.537	1.040	2.270	
	Bias%	4.0	-4.3	7.0	0.0	-11.9	-6.8	6.0	7.0	3.0	≤ ±15%
		-2.8	22.0	5.0	-6.6	-12.0	-12.7	7.0	4.0	13.0	
	n	17									≥ 14
5-Aug-21 / 5	Conc	0.0042	0.011	0.024	0.056	0.092	0.225	0.560	0.884	2.200	
	µM	0.0058	0.0388*	0.022	0.0365*	0.096	0.224	0.570	0.911	2.270	
	Bias%	-16.4	5.0	-2.3	12.0	-7.7	-10.0	12.0	-11.6	10.0	≤ ±15%
		16.0	288.0	-10.6	-27.0	-4.3	-10.5	14.0	-8.9	13.0	
	n	16									≥ 14
Inter-Run	Mean	0.0049	0.010	0.025	0.051	0.092	0.239	0.533	0.975	2.061	
	S.D.	0.0005	0.001	0.003	0.004	0.004	0.022	0.032	0.081	0.141	
	%CV	10.8	7.1	10.9	8.4	4.6	9.3	5.9	8.3	6.9	≤ 15%
	%Bias	-1.9	2.5	1.2	2.5	-8.1	-4.6	6.7	-2.5	3.0	≤ ±15%
	n	9	8	11	11	11	12	11	11	12	

** Did not met acceptance criteria*

Table 32 Calibration Curve Parameters for T4G Calibration Standards in HLM

Run Date	Run Number	Slope	Intercept	R-Squared	LLOQ	ULOQ	Regression Footnote(s)
1-Jul-21	0	1.28	0.0036	0.9973	0.005	2	1/(x*x)
6-Jul-21	1	1.45	0.0049	0.9963	0.005	2	1/(x*x)
9-Jul-21	2	1.27	0.0016	0.9956	0.005	2	1/(x*x)
9-Jul-21	3	1.28	0.0037	0.9956	0.005	2	1/(x*x)
22-Jul-21	4	1.23	0.0017	0.9960	0.005	2	1/(x*x)
5-Aug-21	5	1.31	0.0078	0.9919	0.005	2	1/(x*x)

Table 33 Precision and accuracy of T4G Quality Control

Run Date	Run Number	QC Conc µM	LQC 0.015	MQC 0.1	HQC 1.5	AC
1-Jul-21	0	Conc µM	0.0381* 0.0397*	0.1150 0.141*	1.4 1.5	
		Intra-run %Bias n	138.0 148.0	15.0 18.0 3	-8.9 -3.3	≤ ± 15% ≥ 4
6-Jul-21	1	Conc µM	0.0183 0.0174	0.112 0.139*	1.39 1.28	
		Intra-run %Bias n	15.0 9.0	12.0 39.0* 5	-7.4 -14.5	≤ ± 15% ≥ 4
9-Jul-21	2	Conc µM	0.0159 0.0225*	0.114 0.108	1.28 1.38	
		Intra-run %Bias n	-0.8 41.0	14.0 8.0 5	-14.4 -7.8	≤ ± 15% ≥ 4
14-Jul-21	3	Conc µM	0.0263* 0.0181	0.0858 0.101	1.51 1.66	
		Intra-run %Bias n	64.0 13.0	-14.2 1.0 5	1.0 10.0	≤ ± 15% ≥ 4
22-Jul-21	4	Conc µM	0.0151 0.0151	0.110 0.114	1.69 1.38	
		Intra-run %Bias n	1.0 0.0	10.0 14.0 5	12.0 -8.2	≤ ± 15% ≥ 4
5-Aug-21	5	Conc µM	0.0570* 0.0183	0.112 0.110	1.3 1.32	
		Intra-run %Bias n	256.0 14.0	12.0 10.0 5	-13.5 -12.0	≤ ± 15% ≥ 4
Inter-run[(a)]		Mean S.D. %CV %Bias n	0.0169 0.0015 8.7 12.6 7	0.1074 0.0090 8.4 7.4 9	1.4190 0.1514 10.7 -5.4 10	 ≤ 15% ≤ ± 15% ≥ 7

** Did not met acceptance criteria*
[(a)] Range finding assay QC were not considered

Table 34 T4G Selectivity Effect Test in HLM in the five valid runs

Run Date	Run Number	Analyte Peak Area		
		T4G	T4	Fexofenadine
6-Jul-21	1	No Peak	1.25E+06	1.60E+05
		No Peak	8.80E+05	1.23E+05
		No Peak	8.33E+05	1.29E+05
9-Jul-21	2	No Peak	1.42E+06	1.80E+05
		No Peak	1.38E+06	1.72E+05
		No Peak	1.28E+06	1.68E+05
14-Jul-21	3	No Peak	1.80E+06	1.92E+05
		No Peak	9.24E+05	1.45E+05
		No Peak	1.07E+06	1.52E+05
22-Jul-21	4	No Peak	8.04E+05	1.36E+05
		No Peak	7.16E+05	1.49E+05
		No Peak	7.27E+05	1.57E+05
5-Aug-21	5	No Peak	8.87E+05	1.76E+05
		No Peak	7.74E+05	1.55E+05
		No Peak	8.04E+05	1.58E+05

Table 35 Selectivity Effect Test in HLM in T4G formation without UDPGA

Run Date	Run Number	Analyte Peak Area					
		T4 2 µM		T4 5 µM		T4 10 µM	
		T4G	T4	T4G	T4	T4G	T4
1-Jul-21	0	No Peak	1.79E+06	No Peak	1.65E+06	No Peak	2.61E+06
		No Peak	1.72E+06	No Peak	1.74E+06	No Peak	2.57E+06
		No Peak	1.91E+06	No Peak	1.65E+06	No Peak	2.18E+06
6-Jul-21	1	No Peak	7.96E+05	No Peak	1.02E+06	No Peak	2.71E+06
		No Peak	7.93E+05	No Peak	1.48E+06	No Peak	2.83E+06
		No Peak	7.76E+05	No Peak	1.53E+06	No Peak	N/D
9-Jul-21	2	No Peak	7.56E+05	No Peak	1.82E+06	No Peak	3.14E+06
		No Peak	7.71E+05	No Peak	1.96E+06	No Peak	2.87E+06
		No Peak	6.93E+05	No Peak	2.13E+06	No Peak	4.25E+05
14-Jul-21	3	No Peak	7.11E+05	No Peak	1.25E+06	No Peak	2.01E+06
		No Peak	7.13E+05	No Peak	1.65E+06	No Peak	1.40E+06
		No Peak	6.47E+05	No Peak	1.50E+06	No Peak	1.51E+06
22-Jul-21	4	No Peak	4.84E+05	No Peak	1.42E+06	No Peak	3.51E+06
		No Peak	4.45E+05	No Peak	1.61E+06	No Peak	3.38E+06
		No Peak	5.98E+05	No Peak	1.92E+06	No Peak	4.62E+06
5-Aug-21	5	No Peak	9.76E+05	No Peak	2.32E+06	No Peak	3.91E+06
		No Peak	9.08E+05	No Peak	2.29E+06	No Peak	3.99E+06
		No Peak	1.17E+06	No Peak	2.75E+06	No Peak	4.54E+06

12.10 Tables: experimental results of T3G formation/inhibition

Table 36 Range finding Assay: effect of Mefenamic Acid and No Solvent on the T4G formation

1-Jul-21		**TI**	**TI**	**TI**	**TI**	**TI**	**TI**	**TI**	**TI**	**NC**
ID		**C1**	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**	**C1**
Conc µM		**500.0**	**200.0**	**50.0**	**20.0**	**5.0**	**2.5**	**0.2**	**0.02**	**500.0**
T4 2 µM										
% activities vs Solvent	r1	8.8	35.2	67.0	97.0	93.8	95.7	104.0	114.9	113.6
	r2	8.8	28.9	74.7	92.6	97.7	79.8	76.0	118.1	100.2
	r3	8.4	30.4	72.8	90.0	98.9	93.2	113.6	113.0	108.5
	Avg	8.7	31.5	71.5	93.2	96.8	89.6	97.9	115.3	107.4
	CV %	3.0	10.6	5.6	3.8	2.7	9.6	20.0	2.2	6.3
% Inhibition	**Avg**	**91.3**	**68.5**	**28.5**	**6.8**	**3.2**	**10.4**	**2.1**	**-15.3**	**-7.4**
T4 5 µM										
% activities vs Solvent	r1	8.6	28.3	82.0	94.7	97.7	97.7	109.0	114.3	108.3
	r2	7.6	34.4	78.9	106.0	109.8	98.5	113.5	110.5	105.3
	r3	5.3	34.4	56.8	85.0	103.8	114.3	105.3	109.0	90.2
	Avg	7.2	32.4	72.6	95.2	103.8	103.5	109.3	111.3	101.3
	CV %	23.7	10.8	18.9	11.1	5.8	9.0	3.8	2.4	9.5
% Inhibition	**Avg**	**92.8**	**67.6**	**27.4**	**4.8**	**-3.8**	**-3.5**	**-9.3**	**-11.3**	**-1.3**
T4 10 µM										
% activities vs Solvent	r1	12.9	40.6	81.1	100.1	152.9	95.7	112.6	108.6	101.0
	r2	14.5	35.9	76.7	97.9	141.4	98.8	103.2	93.1	105.5
	r3	15.2	36.9	76.7	105.5	139.6	93.5	105.5	105.9	98.8
	Avg	14.2	37.8	78.1	101.2	144.6	96.0	107.1	102.5	101.8
	CV %	8.1	6.6	3.3	3.8	5.0	2.8	4.5	8.1	3.3
% Inhibition	**Avg**	**85.8**	**62.2**	**21.9**	**-1.2**	**-44.6**	**4.0**	**-7.1**	**-2.5**	**-1.8**

Table 37 Main Inhibition Assay: T4G formation in the "Solvent" in the five valid runs

Run Date / Run N°	T4G Concentrations µM			T4G Formation Rate			AC
	T4	2 µM	5 µM	10 µM	2 µM	5 µM	10 µM	
6-Jul-21 / 1	r1	0.239	0.698	1.33	1.328	3.878	7.389	
	r2	0.248	0.645	1.33	1.378	3.583	7.389	**≥ 2.000** [(a)]
	r3	0.222	0.696	0.969	1.233	3.867	5.383	
	Mean	0.2363	0.680	1.210	1.313	3.776	6.720	**≥ 2.000** [(a)]
	SD	0.0132	0.030	0.208	0.073	0.167	1.158	
	CV %	5.6	4.4	17.2	5.6	4.4	17.2	**≤20%**
9-Jul-21 / 2	r1	0.194	0.497	1.17	1.078	2.761	6.500	
	r2	0.18	0.513	1.04	1.000	2.850	5.778	**≥ 2.000** [(a)]
	r3	0.197	0.493	0.927	1.094	2.739	5.150	
	Mean	0.1903	0.501	1.046	1.057	2.783	5.809	**≥ 2.000** [(a)]
	SD	0.0091	0.011	0.122	0.050	0.059	0.676	
	CV %	4.8	2.1	11.6	4.8	2.1	11.6	**≤20%**
14-Jul-21 / 3	r1	0.215	0.755	1.32	1.194	4.194	7.333	
	r2	0.213	0.76	1.31	1.183	4.222	7.278	**≥ 2.000** [(a)]
	r3	0.228	0.821	1.09	1.267	4.561	6.056	
	Mean	0.2187	0.779	1.240	1.215	4.326	6.889	**≥ 2.000** [(a)]
	SD	0.0081	0.037	0.130	0.045	0.204	0.722	
	CV %	3.7	4.7	10.5	3.7	4.7	10.5	**≤20%**
22-Jul-21 / 4	r1	0.0961	0.328	0.849	0.534	1.822	4.717	
	r2	0.0938	0.34	0.805	0.521	1.889	4.472	**≥ 2.000** [(a)]
	r3	0.0843	0.368	0.603	0.468	2.044	3.350	
	Mean	0.0914*	0.345*	0.752*	0.508*	1.919*	4.180*	**≥ 2.000** [(a)]
	SD	0.0063	0.021	0.131	0.035	0.114	0.729	
	CV %	6.8	5.9	17.4	6.8	5.9	17.4	**≤20%**
5-Aug-21 / 5	r1	0.194	0.553	1.57	1.078	3.072	8.722	
	r2	0.182	0.665	1.17	1.011	3.694	6.500	**≥ 2.000** [(a)]
	r3	0.189	0.577	1.17	1.050	3.206	6.500	
	Mean	0.1883	0.598	1.303	1.046	3.324	7.241	**≥ 2.000** [(a)]
	SD	0.0060	0.059	0.231	0.033	0.328	1.283	
	CV %	3.2	9.9	17.7	3.2	9.9	17.7	**≤20%**
Inter-run	Mean	0.2084	0.512	1.200	1.1579	3.552	6.665	**≥ 2.000** [(a)]
	SD	0.0232	0.118	0.110	0.1289	0.656	0.610	
	CV %	11.1	23.1	9.2	11.1	18.5	9.2	**≤20%**

[(a)] *Acceptance Criteria: ≥ 2.000 pmol/mg protein/min of T4G when T4 was incubated at 10 µM*

** Did not met acceptance criteria*

Table 38 Main Inhibition Assay: T4G formation in the "No Solvent" in the five valid runs

Run Date/ Run N°		T4G Concentrations µM			T4G % activity vs solvent			AC
	T4	2 µM	5 µM	10 µM	2 µM	5 µM	10 µM	
6-Jul-21 / 1	r1	0.246	0.765	1.19	104.1	112.6	98.4	
	r2	0.251	0.712	1.22	106.2	104.8	100.9	80-120% [a]
	r3	0.22	0.726	1.01	93.1	106.8	83.5	
	Mean	0.2390	0.734	1.140	101.1	108.0	94.2	80-120% [a]
	SD	0.0166	0.027	0.114	7.0	4.0	9.4	
	CV %	7.0	3.7	10.0	7.0	3.7	10.0	≤20%
9-Jul-21 / 2	r1	0.185	0.442	1	97.2	88.2	95.6	
	r2	0.213	0.465	0.921	111.9	92.8	88.1	80-120% [a]
	r3	0.192	0.572	0.902	100.9	114.2	86.3	
	Mean	0.1967	0.493	0.941	103.3	98.4	90.0	80-120% [a]
	SD	0.0146	0.069	0.052	7.7	13.8	5.0	
	CV %	7.4	14.1	5.5	7.4	14.1	5.5	≤20%
14-Jul-21 / 3	r1	0.209	0.772	1.25	95.6	99.1	100.8	
	r2	0.212	0.735	1.35	97.0	94.4	108.9	80-120% [a]
	r3	0.201	0.866	1.18	91.9	111.2	95.2	
	Mean	0.2073	0.791	1.260	94.8	101.6	101.6	80-120% [a]
	SD	0.0057	0.068	0.085	2.6	8.7	6.9	
	CV %	2.7	8.5	6.8	2.7	8.5	6.8	≤20%
22-Jul-21 / 4	r1	0.177	0.657	1.45	101.9[b]	103.0[b]	103.8[b]	
	r2	0.163	0.613	1.47	104.2[b]	100.0[b]	96.2[b]	80-120% [a]
	r3	0.174	0.662	1.35	93.9[b]	97.1[b]	100.0[b]	
	Mean	0.1713	0.644	1.423	100.0[b]	100.0[b]	100.0[b]	80-120% [a]
	SD	0.0074	0.027	0.064	5.4	3.0	3.8	
	CV %	4.3	4.2	4.5	5.4	3.0	3.8	≤20%
5-Aug-21 / 5	r1	0.201	0.677	1.57	106.7	113.1	120.5	
	r2	0.232	0.549	1.33	123.2	91.8	102.0	80-120% [a]
	r3	0.18	0.507	1.13	95.6	84.7	86.7	
	Mean	0.2043	0.578	1.343	108.5	96.5	103.1	80-120% [a]
	SD	0.0262	0.089	0.220	13.9	14.8	16.9	
	CV %	12.8	15.3	16.4	12.8	15.3	16.4	≤20%
Inter-run	Mean	0.2037	0.648	1.222	101.9	101.1	97.2	80-120%
	SD	0.0243	0.119	0.189	5.7	5.0	6.2	
	CV %	11.9	18.4	15.4	5.6	5.0	6.4	≤20%

[a] *Acceptance Criteria: the percentage activity versus solvent were 80 – 120 %*
[b] *% Activity values were calculated versus mean of No solvent*

Table 39 Main Inhibition Assay: T4G formation in the "Negative Control" in the five valid runs

Run Date/ Run N°	T4G Concentrations µM			T4G % activity vs solvent			AC
	T4	2 µM	5 µM	10 µM	2 µM	5 µM	10 µM	
6-Jul-21 / 1	r1	0.273	0.811	1.23	115.5	119.3	101.7	
	r2	0.247	0.794	1.29	104.5	116.8	106.6	80-120% [a]
	r3	0.24	0.723	1.4	101.6	106.4	115.7	
	Mean	0.2533	0.776	1.307	107.2	114.2	108.0	80-120% [a]
	SD	0.0174	0.047	0.086	7.4	6.9	7.1	
	CV %	6.9	6.0	6.6	6.9	6.0	6.6	≤20%
9-Jul-21 / 2	r1	0.231	0.512	1.16	121.4	102.2	110.9	
	r2	0.227	0.502	0.967	119.3	100.2	92.5	80-120% [a]
	r3	0.219	0.453	0.837	115.1	90.4	80.0	
	Mean	0.2257	0.489	0.988	118.6	97.6	94.5	80-120% [a]
	SD	0.0061	0.032	0.163	3.2	6.3	15.5	
	CV %	2.7	6.5	16.4	2.7	6.5	16.4	≤20%
14-Jul-21 / 3	r1	0.251	0.758	1.37	N/D*	97.3	110.5	
	r2	0.28	0.759	1.28	128.0	97.5	103.2	80-120% [a]
	r3	0.284	0.741	1.44	129.9	95.2	116.1	
	Mean	0.2717	0.753	1.363	129.0	96.7	109.9	80-120% [a]
	SD	0.0180	0.010	0.080	1.3	1.3	6.5	
	CV %	6.6	1.3	5.9	1.0	1.3	5.9	≤20%
22-Jul-21 / 4	r1	0.209	0.763	1.65	122.0[b]	118.5[b]	115.9[b]	
	r2	0.204	0.76	1.53	119.1[b]	118.0[b]	107.5[b]	80-120% [a]
	r3	0.191	0.738	1.59	111.5[b]	114.6[b]	111.7[b]	
	Mean	0.2013	0.754	1.590	117.5[b]	117.0[b]	111.7[b]	80-120% [a]
	SD	0.0093	0.014	0.060	5.4	2.1	4.2	
	CV %	4.6	1.8	3.8	4.6	1.8	3.8	≤20%
5-Aug-21 / 5	r1	0.205	0.618	1.38	108.8	103.3	105.9	
	r2	0.189	0.775	1.27	100.4	129.5	97.4	80-120% [a]
	r3	0.173	0.671	1.24	91.9	112.1	95.1	
	Mean	0.1890	0.688	1.297	100.4	115.0	99.5	80-120% [a]
	SD	0.0160	0.080	0.074	8.5	13.3	5.7	
	CV %	8.5	11.6	5.7	8.5	11.6	5.7	≤20%
Inter-run	Mean	0.2329	0.693	1.309	117.2	108.3	104.7	80-120%
	SD	0.0315	0.119	0.215	13.4	10.3	7.4	
	CV %	13.5	17.2	16.4	11.4	9.5	7.1	≤20%

[a] *Acceptance Criteria: the percentage activity versus solvent were 80 – 120 %*
[b] *% Activity values were calculated versus mean of No solvent*

Table 40 Main Inhibition Assay: T4G formation in the Test Item in the five valid runs (T4 2 µM)

Run Date/ Run N°	T4 2 µM % Activity vs Solvent								AC
	Mefenamic Acid	**C1 500.0**	**C2 320.0**	**C3 200.0**	**C4 160.0**	**C5 80.0**	**C6 50.0**	**C7 20.0**	**C8 5.0**	
6-Jul-21 / 1	r1	9.8	22.6	36.9	44.4	63.5	56.7	95.6	109.6	
	r2	11.6	22.9	31.4	45.3	60.9	62.6	88.9	101.1	
	r3	11.9	18.7	31.9	44.4	58.0	59.2	83.8	98.6	
	Mean	11.1	21.4	33.4	44.7	60.8	59.5	89.4	103.1	
	SD	1.1	2.3	3.1	0.5	2.8	3.0	5.9	5.8	
	CV%	10.3	10.9	9.2	1.1	4.5	5.0	6.6	5.6	≤20%
9-Jul-21 / 2	r1	15.4	28.6	38.8	43.6	68.8	69.4	93.5	104.6	
	r2	13.7	25.8	37.1	44.0	68.3	69.9	N/D*	102.5	
	r3	12.1	23.7	31.8	42.0	57.3	61.5	82.0	99.3	
	Mean	13.7	26.1	35.9	43.2	64.8	66.9	87.7	102.1	
	SD	1.6	2.5	3.6	1.1	6.5	4.7	8.2	2.6	
	CV %	11.9	9.4	10.1	2.5	10.1	7.0	9.3	2.6	≤20%
14-Jul-21 / 3	r1	10.2	24.6	38.1	44.2	62.2	60.4	97.0	117.1	
	r2	10.5	23.0	37.5	44.9	59.5	57.2	108.4	100.6	
	r3	10.8	20.7	35.8	40.8	54.4	50.3	83.2	115.2	
	Mean	10.5	22.7	37.1	43.3	58.7	55.9	96.2	111.0	
	SD	0.3	2.0	1.2	2.2	3.9	5.1	12.6	9.0	
	CV %	2.8	8.6	3.2	5.0	6.7	9.2	13.1	8.1	≤20%
22-Jul-21 / 4[(b)]	r1	8.4	17.6	37.8	45.0	72.4	73.5	108.6	134.2	
	r2	11.3	13.5	38.0	47.5	74.1	73.0	105.6	123.2	
	r3	9.6	12.1	37.5	39.6	71.2	55.5	91.6	125.5	
	Mean	9.8	14.4*	37.7	44.0	72.6	67.3	101.9	127.6	
	SD	1.5	2.8	0.3	4.1	1.5	10.2	9.0	5.8	
	CV %	15.0	19.6	0.7	9.2	2.0	15.2	8.9	4.6	≤20%
5-Aug-21 / 5	r1	9.2	19.7	27.4	39.9	53.6	61.6	95.6	102.5	
	r2	10.6	17.6	25.0	38.8	52.8	53.0	108.3	104.6	
	r3	12.1	17.6	26.9	33.4	45.1	70.1	85.0	140.7	
	Mean	10.7	18.3	26.4	37.4	50.5	61.6	96.3	115.9	
	SD	1.4	1.2	1.3	3.5	4.7	8.5	11.7	21.5	
	CV %	13.5	6.5	4.9	9.3	9.3	13.9	12.1	18.5	≤20%
Inter-run	Mean	11.2	20.6	34.1	42.5	61.5	62.3	94.3	111.9	
	SD	1.5	4.4	4.6	2.9	8.1	4.9	5.8	10.5	
	CV %	13.6	21.6	13.5	6.9	13.2	7.8	6.1	9.3	≤20%

[(b)] *% Activity values were calculated versus mean of No solvent*

** Did not met acceptance criteria*

Table 41 Main Inhibition Assay: T4G formation in the test item in the five valid runs (T4 5 µM)

Run Date/ Run N°	T4 5 µM % Activity vs Solvent									AC
	Mefenamic Acid	C1 500.0	C2 320.0	C3 200.0	C4 160.0	C5 80.0	C6 50.0	C7 20.0	C8 5.0	
6-Jul-21 / 1	r1	9.5	18.5	31.8	45.6	56.8	70.0	91.2	107.4	
	r2	11.4	18.4	37.2	49.7	54.9	64.6	83.4	96.7	
	r3	11.3	22.7	35.5	45.9	64.0	64.4	87.0	102.1	
	Mean	10.8	19.9	34.8	47.1	58.6	66.4	87.2	102.1	
	SD	1.1	2.4	2.8	2.3	4.8	3.2	3.9	5.4	
	CV %	9.8	12.2	8.0	4.9	8.2	4.8	4.5	5.3	≤20%
9-Jul-21 / 2	r1	11.7	23.4	36.3	37.9	57.5	65.1	97.6	110.2	
	r2	12.7	25.9	32.9	40.7	63.1	73.7	99.2	112.6	
	r3	11.2	20.8	31.9	38.7	64.1	63.3	94.6	106.8	
	Mean	11.9	23.4	33.7	39.1	61.5	67.3	97.1	109.8	
	SD	0.7	2.6	2.3	1.4	3.6	5.5	2.3	2.9	
	CV %	6.2	11.1	6.8	3.7	5.8	8.2	2.4	2.6	≤20%
14-Jul-21 / 3	r1	8.7	16.2	27.5	32.4	51.5	68.6	79.8	114.0	
	r2	9.1	18.6	33.0	37.1	53.9	64.2	87.7	100.3	
	r3	9.9	19.3	33.5	42.6	57.4	78.0	89.0	82.8	
	Mean	9.2	18.0	31.3	37.4	54.3	70.2	85.5	99.1	
	SD	0.6	1.6	3.3	5.1	3.0	7.0	5.0	15.6	
	CV %	6.9	9.0	10.7	13.8	5.5	10.0	5.9	15.8	≤20%
22-Jul-21 / 4[(b)]	r1	8.8	12.5	30.1	41.8	62.6	61.2	96.4	121.7	
	r2	8.4	12.9	30.4	40.4	61.3	54.8	95.5	111.8	
	r3	8.7	13.8	32.0	41.6	64.8	70.3	98.4	125.5	
	Mean	8.6	13.1	30.8	41.3	62.9	62.1	96.8	119.7	
	SD	0.2	0.6	1.0	0.8	1.7	7.8	1.5	7.1	
	CV %	1.9	5.0	3.2	1.9	2.7	12.6	1.6	5.9	≤20%
5-Aug-21 / 5	r1	9.9	18.7	31.8	50.6	66.7	84.6	91.4	115.7	
	r2	12.2	17.9	30.8	40.9	61.7	60.8	87.1	108.1	
	r3	10.0	19.9	29.2	37.3	58.8	62.3	87.4	96.9	
	Mean	10.7	18.8	30.6	43.0	62.4	69.2	88.6	106.9	
	SD	1.3	1.0	1.3	6.9	4.0	13.3	2.4	9.4	
	CV %	12.2	5.4	4.1	16.1	6.4	19.2	2.7	8.8	≤20%
Inter-run	Mean	10.2	18.6	32.3	41.6	59.9	67.1	91.1	107.5	
	SD	1.3	3.7	1.9	3.7	3.6	3.2	5.5	8.0	
	CV %	12.7	19.9	5.9	9.0	6.0	4.7	6.1	7.4	≤20%

[(b)] *% A values were calculated versus mean of No solvent*

Table 42 Main Inhibition Assay: T4G formation in the test item in the five valid runs (T4 10 µM)

Run Date/ Run N°	T4 10 µM % Activity vs Solvent									AC
	Mefenamic Acid	C1 500.0	C2 320.0	C3 200.0	C4 160.0	C5 80.0	C6 50.0	C7 20.0	C8 5.0	
6-Jul-21 / 1	r1	12.9	25.0	40.7	54.9	65.4	78.5	93.4	100.9	
	r2	11.4	23.3	42.2	49.4	66.5	80.4	100.0	102.5	
	r3	11.6	20.7	40.9	39.6	60.2	70.0	101.7	97.5	
	Mean	12.0	23.0	41.3	47.9	64.0	76.3	98.4	100.3	
	SD	0.8	2.2	0.8	7.7	3.4	5.5	4.4	2.5	
	CV %	6.8	9.4	2.0	16.1	5.2	7.3	4.4	2.5	≤20%
9-Jul-21 / 2	r1	12.7	25.7	36.5	42.9	58.5	67.2	108.1	110.9	
	r2	11.9	20.2	31.2	34.8	56.5	65.9	94.4	90.7	
	r3	3.5*	N/D*	25.5	33.1	49.3	48.9	N/D*	89.0	
	Mean	12.3	23.0	31.1	36.9	54.8	60.7	101.2	96.9	
	SD	0.6	3.9	5.5	5.3	4.9	10.2	9.7	12.2	
	CV %	5.0	17.1	17.7	14.2	8.9	16.9	9.6	12.6	≤20%
14-Jul-21 / 3	r1	15.7	23.3	38.9	44.2	67.7	93.5	95.2	104.0	
	r2	12.5	21.9	38.8	49.4	70.5	63.6	87.1	115.3	
	r3	N/D*	18.3	34.9	43.6	N/D*	73.3	77.7	N/D*	
	Mean	14.1	21.2	37.5	45.7	69.1	76.8	86.6	109.7	
	SD	2.3	2.6	2.3	3.2	2.0	15.3	8.8	8.0	
	CV %	16.2	12.2	6.0	6.9	2.9	19.9	10.1	7.3	≤20%
22-Jul-21 / 4[(b)]	r1	10.4	15.9	33.9	43.5	67.7	62.6	99.8	111.0	
	r2	8.7	14.0	33.0	40.5	63.2	61.7	112.4	114.5	
	r3	9.8	15.2	33.6	49.5	70.2	61.5	101.2	130.0	
	Mean	9.6	15.0	33.5	44.5	67.0	61.9	104.4	118.5	
	SD	0.9	1.0	0.5	4.5	3.6	0.6	6.9	10.1	
	CV %	8.9	6.4	1.4	10.2	5.3	0.9	6.6	8.5	≤20%
5-Aug-21 / 5	r1	10.7	17.4	32.2	43.6	65.3	72.6	101.3	117.4	
	r2	9.7	18.4	32.3	46.3	60.8	66.5	97.4	108.2	
	r3	9.2	14.0	26.9	43.7	54.5	58.3	77.5	81.3	
	Mean	9.9	16.6	30.5	44.5	60.2	65.8	92.1	102.3	
	SD	0.8	2.3	3.1	1.6	5.4	7.2	12.8	18.7	
	CV %	8.0	13.8	10.3	3.5	9.0	10.9	13.9	18.3	≤20%
Inter-run	Mean	11.6	19.8	34.8	43.9	63.0	68.3	96.6	105.5	
	SD	1.9	3.7	4.6	4.1	5.7	7.8	7.2	8.6	
	CV %	16.0	18.7	13.2	9.4	9.0	11.4	7.4	8.2	≤20%

[(b)] *% Activity values were calculated versus mean of No solvent*

Table 43 Main Inhibition Assay: IC50 Curve of T4G in presence of Test Item in the five valid runs

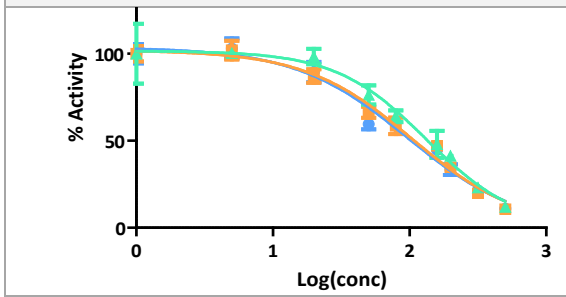
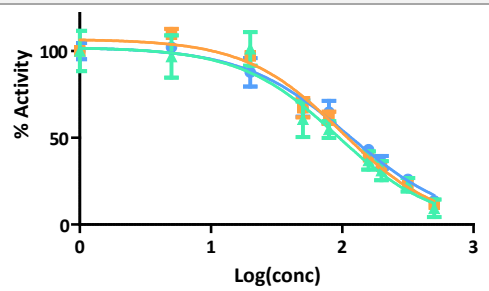
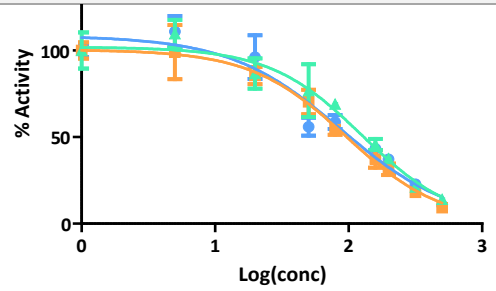
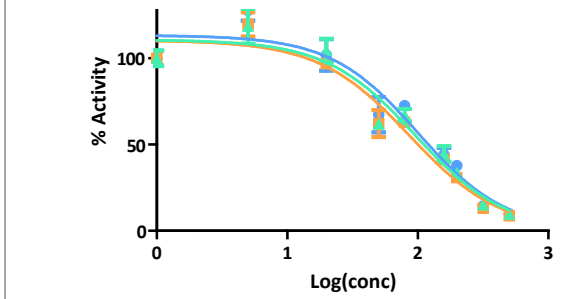
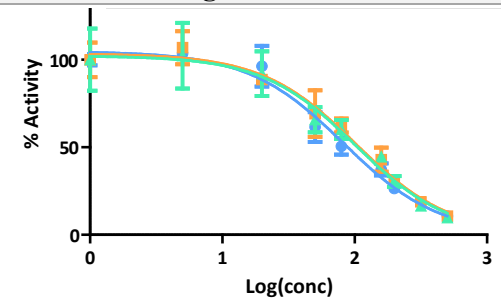
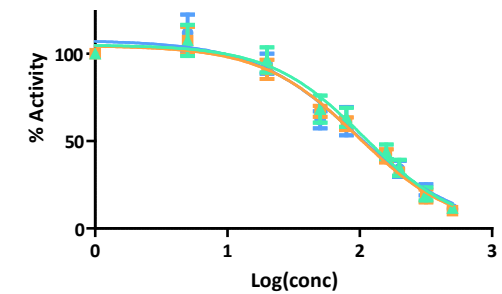
6-Jul-21 / RUN 1					**9-Jul-21 / RUN 2**					**14-Jul-21 / RUN 3**				
														
T4G	**2 µM**	**5 µM**	**10 µM**	**AC**	**T4G**	**2 µM**	**5 µM**	**10 µM**	**AC**	**T4G**	**2 µM**	**5 µM**	**10 µM**	**AC**
IC50	96.52	104.6	132.5		IC50	112.6	98.71	97.74		IC50	88.32	96.22	126.1	
R-square	0.9695	0.9776	0.9609	≥ 0.9000	R-square	0.9790	0.9809	0.9870	≥ 0.9000	R-square	0.9394	0.9705	126.1	≥ 0.9000
n	27	27	27	≥ 20	n	26	27	25	≥ 20	n	27	27	24	≥ 20
22-Jul-21 / RUN 4					**5-Aug-21 / RUN 5**					**Inter-Run**				
														
T4G	**2 µM**	**5 µM**	**10 µM**	**AC**	**T4G**	**2 µM**	**5 µM**	**10 µM**	**AC**	**T4G**	**2 µM**	**5 µM**	**10 µM**	**AC**
IC50	100.6	86.29	95.63		IC50	82.19	102.5	101.6		IC50	93.74	97.15	106.9	
R-square	0.9305	0.9553	0.9426	≥ 0.9000	R-square	0.9678	0.9613	0.9294	≥ 0.9000	R-square	0.9537	0.9779	0.9655	≥ 0.9000
n	27	27	27	≥ 20	n	27	27	27	≥ 20	n	44	45	45	≥ 33

Table 44 Main Inhibition Assay: Summary inhibition on the T4G formation

T4G % Inhibition vs Solvent									
ID	**TI C1**	**TI C2**	**TI C3**	**TI C4**	**TI C5**	**TI C6**	**TI C7**	**TI C8**	**NC C1**
Conc µM	**500.0**	**320.0**	**200.0**	**160.0**	**80.0**	**50.0**	**20.0**	**5.0**	**500.0**
T4 2 µM									
Run 1	88.9	78.6	66.6	55.3	39.2	40.5	10.6	-3.1	-7.2
Run 2	86.3	73.9	64.1	56.8	35.2	33.1	12.3	-2.1	-18.6
Run 3	89.5	77.3	62.9	56.7	41.3	44.1	3.8	-11.0	-24.2
Run 4	90.2	85.6	62.3	56.0	27.4	32.7	-1.9	-27.6	-31.1
Run 5	89.3	81.7	73.6	62.6	49.5	38.4	3.7	-15.9	-0.4
Inter Run	**88.8**	**79.4**	**65.9**	**57.5**	**38.5**	**37.7**	**5.7**	**-11.9**	**-16.3**
T4 5 µM									
Run 1	89.2	80.1	65.2	52.9	41.4	33.6	12.8	-2.1	-14.2
Run 2	88.1	76.6	66.3	60.9	38.5	32.7	2.9	-9.8	2.4
Run 3	90.8	82.0	68.7	62.6	45.7	29.8	14.5	0.9	3.3
Run 4	91.4	86.9	69.2	58.7	37.1	37.9	3.2	-19.7	-18.1
Run 5	89.3	81.2	69.4	57.0	37.6	30.8	11.4	-6.9	-15.0
Inter Run	**89.8**	**81.4**	**67.7**	**58.4**	**40.1**	**32.9**	**8.9**	**-7.5**	**-8.3**
T4 10 µM									
Run 1	88.0	77.0	58.7	52.1	36.0	23.7	1.6	-0.3	-8.0
Run 2	87.7	77.0	68.9	63.1	45.2	39.3	-1.2	3.1	5.5
Run 3	85.9	78.8	62.5	54.3	30.9	23.2	13.4	-9.7	-9.9
Run 4	90.4	85.0	66.5	55.5	33.0	38.1	-4.4	-18.5	-11.7
Run 5	89.8	83.4	69.5	55.5	36.9	34.2	7.9	-12.8	0.5
Inter Run	**88.4**	**80.2**	**65.2**	**56.1**	**36.4**	**31.7**	**3.4**	**-7.6**	**-4.7**

Table 45 Summary T4G IC50 data

IC50 T4G	T4 2 µM	T4 5 µM	T4 10 µM
Run 1	96.52	104.60	132.50
Run 2	112.60	98.71	91.74
Run 3	88.32	96.22	126.10
Run 4	100.60	86.29	95.63
Run 5	82.19	102.50	101.60
Avg	**96.05**	**97.66**	**109.51**
CV%	**12.17**	**7.31**	**16.93**
Inter-Run	93.74	97.15	106.9

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14 ABBREVIATIONS

CAS	Chemical Abstracts Service
CoA	Certificate of Analysis
DMSO	Dimethyl sulfoxide
e.g.	For example
GLP	Good Laboratory Practice
h	Hour (s)
H_2O	Ultrapure or deionised water
HLM	Human Liver Microsomes
HPLC	High-performance liquid chromatography
ISTD	Internal Standard
LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
LLOQ	Lower limit of quantitation
MeOH	Methanol
min	Minute(s)
MU	4-Methylumbelliferone
MW	Molecular weight
N/D	Not Detected
NC	Negative Control
PC	Positive Control
OECD	Economic Co-operation and Development
QC	Quality control
RT	Room temperature
SS	Stock solution
T3	L-Thyroxine
T3G	L-Thyroxine glucuronide
T4	3,3',5-Triiodo-L- thyronine
T4G	3,3',5-Triiodo-L- thyronine glucuronide
TI	Test Item
UGT	UDP-glucuronosyltransferases
UDPGA	Uridine 5′-diphospho-glucuronic acid
ULOQ	Upper limit of quantitation
v/v	Volume per volume
WS	Working solution