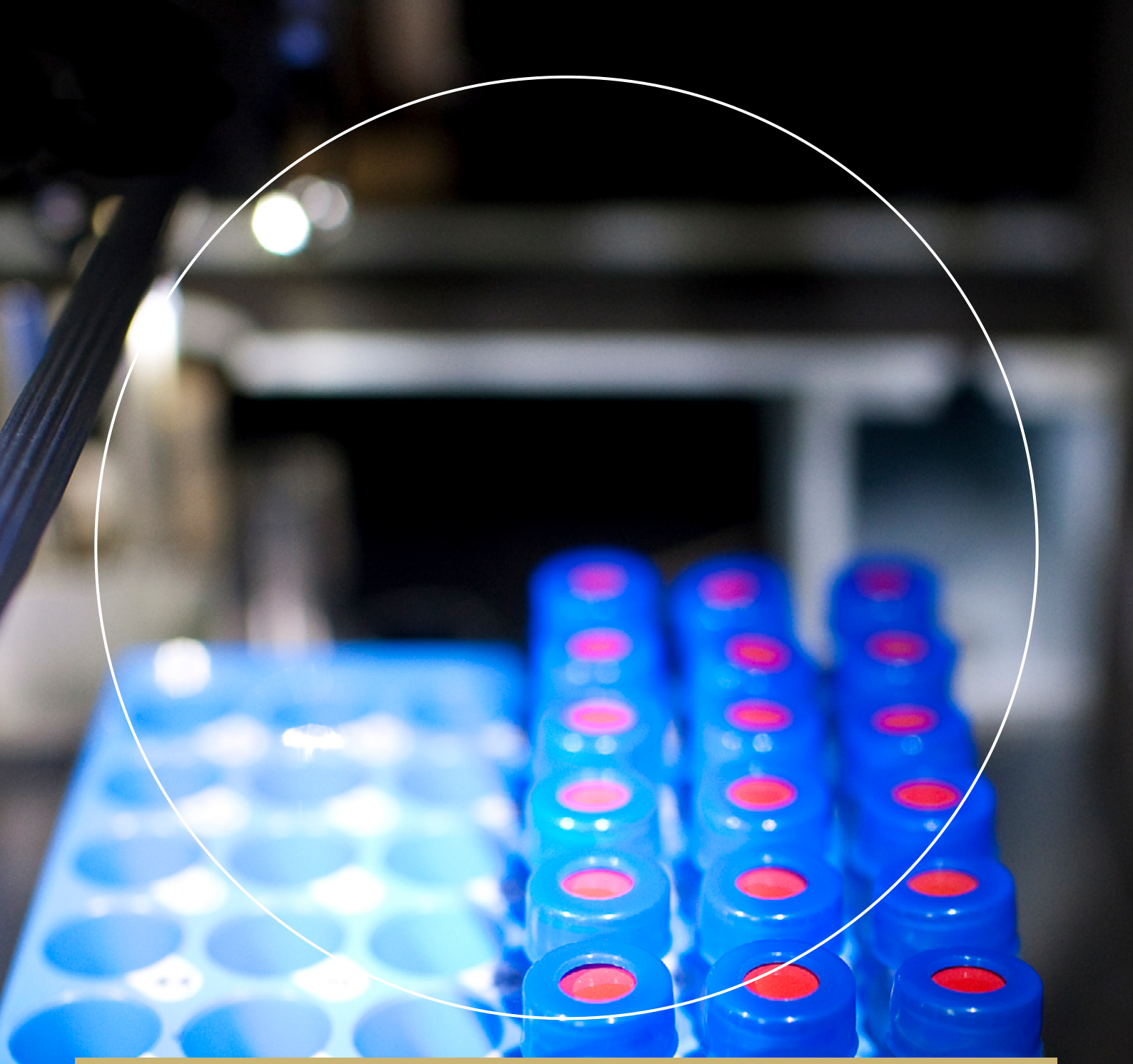

Developing an in vitro assay for thyroid hormone sulphation in primary human hepatocytes

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This research has been carried out by Wageningen Food Safety Research, institute within the legal entity Wageningen Research Foundation subsidised by the Dutch Ministry of Infrastructure and Water Management – Directorate-General for the Environment and International Affairs.

Wageningen, 21 November 2025

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Contents

Summary

English
The Ministry of Infrastructure and Water Management – Directorate-General for the Environment and International Affairs aims to increase the availability of accepted test methods for characterizing endocrine-disrupting substances. An important focus in this regard is the development and refinement of animal-free methods that provide insight into potential disruption of the endocrine system indicating potential developmental neurotoxicity. In the present study, an *in vitro* method was explored that may be suitable for testing disturbances in thyroid hormone metabolism. Primary human hepatocytes were used to investigate the induction of thyroid hormone sulphation, with a specific focus on testing positive controls as a first step toward validation. For this purpose, an analytical method was first developed to quantify the thyroid hormones thyroxine (T4) and triiodothyronine (T3) and their metabolites, T4-sulphate, T4-glucuronide, and T3-sulphate. Subsequently, the usability of the hepatocytes was assessed by examining cell viability and the sulphation activity of T4 and T3 under non-stimulated conditions. Finally, a set of candidate compounds was included as positive controls to induce sulphation activity in the hepatocytes. Although the hepatocytes demonstrated sulphation activity for T4 and T3, the selected candidate compounds were not able to significantly increase this activity. In the future, further investigation of candidate compounds for positive controls is required, with particular attention to improving the experimental conditions through the use of Matrigel.

Nederlands
Het Ministerie van Infrastructuur en Waterstaat – Directoraat-generaal Milieu en Internationaal wil de beschikbaarheid van geaccepteerde testmethoden om hormoonverstorende stoffen te karakteriseren, vergroten. Een belangrijk aandachtspunt daarbij is het ontwikkelen en verfijnen van dierproefvrije methoden die inzicht geven in mogelijke verstoring van het hormoonsysteem welke mogelijk duidt op ontwikkelingsneurotoxiciteit. In het onderzoek dat hier is uitgevoerd is er gekeken naar een *in vitro* methode die in aanmerking kan komen om verstoringen in het metabolisme van schildklierhormonen te testen. Primaire humane hepatocyten zijn gebruikt om naar de inductie te kijken van de sulfatering van schildklierhormonen waarbij er specifiek is ingezet op het testen van positieve controles als eerste stap richting validatie. Daarvoor is er eerst een analytische methode ontwikkeld om schildklierhormonen thyroxine (T4) en triiodothyronine (T3) en hun metabolieten, T4-sulfaat, T4-glucuronide en T3-sulfaat, te kunnen kwantificeren. Daarna is de bruikbaarheid van de hepatocyten getest waarbij gekeken is naar celviabiliteit en sulfateringsactiviteit van T4 en T3 onder niet-gestimuleerde omstandigheden. Als laatste is er een set aan stofkandidaten meegenomen als positieve controle om de sulfateringsactiviteit van de hepatocyten induceren. Alhoewel de hepatocyten sulfateringsactiviteit laten zien voor T4 en T3, waren de gekozen stofkandidaten niet in staat om de sulfateringsactiviteit significant te verhogen. In de toekomst dient er verder gekeken te worden naar stofkandidaten voor positieve controles en in het bijzonder naar het verbeteren van de experimentele condities door gebruik te maken van Matrigel.

1 Introduction

The Ministry of Infrastructure and Water Management – Directorate-General for the Environment and International Affairs aims to increase the availability of accepted test methods for characterizing endocrine-disrupting substances. An important focus in this regard is the development and refinement of animal-free methods that provide insight into potential disruption of the endocrine system. The Organisation for Economic Co-operation and Development (OECD) assessed an in vitro battery (IVB) for developmental neurotoxicity (DNT), which are described in the Initial Recommendations on Evaluation of Data from the Developmental Neurotoxicity (DNT) In-Vitro Testing Battery (oecd.org). Their assessment revealed that test methods linking endocrine activity with DNT are missing. One particular endocrine activity is the disruption of the thyroid hormone system. Given its critical role in DNT, there is considerable international attention—both regulatory and scientific—on thyroid hormone system disruption. Nevertheless, the DNT IVB provides limited focus on testing for such disruption, and appropriate assays are still lacking. The details are further elaborated upon below.

Thyroid hormones (THs) are pivotal in the development of the brain and central nervous system in the unborn child (Giordano & Costa, 2012; Patel et al., 2011). THs are involved in the maturation of the brain during gestation through regulation of genes responsible for myelination and differentiation of neuronal and glial cells. A multifaceted process is required for the THs to reach the foetal brain, which includes 1) expression of thyroid hormone receptors (THRs), 2) transport of materno-foetal THs and iodide, 3) a complex system of endocrine feedback via the hypothalamic-pituitary-thyroid (HPT) axis (Figure 1) and 4) thyroid hormone metabolism by deiodinase enzymes allowing sustained THs basal levels. This means that the role of THs is comprehensive and complex and disturbances can occur at several levels in neurodevelopment.

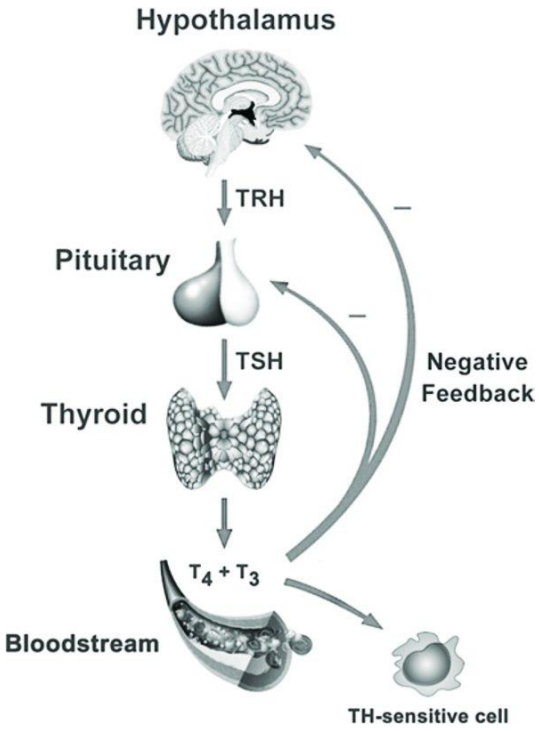

Figure 1 *Hypothalamic pituitary thyroid (HPT) axis.*

There are several enzymes involved in the TH metabolism, among others deiodinases. Deiodinases (D1, D2 and D3) remove iodide from the TH structure according to an inner ring deiodination (IRD) or outer ring deiodination (ORD) where thyroxine (T4), inactive TH, can be degraded to triiodothyronine (T3) via ORD, the active TH that can bind to the THR, or to reverse triiodothyronine (rT3) via IRD, which is inactive for the THR. Both T3 and rT3 can be further degraded to 3,5-diiodo-L-thyronine (T2) either via IRD or ORD. Deiodinase are present in liver, kidney, thyroid gland, pituitary gland, brain and placenta, either found in the plasma membrane (D1 and D3) or the endoplasmic reticulum (D2) (Figure 2) (Patel et al., 2011; Visser, 1988).

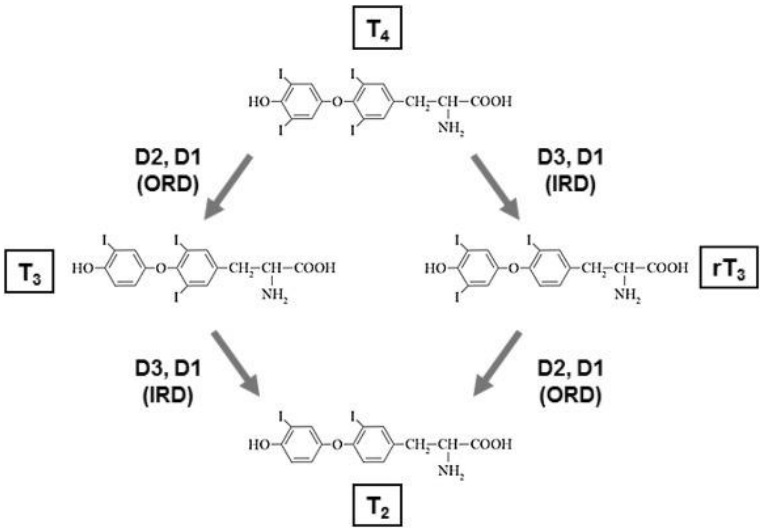

Figure 2 *Deiodination of thyroid hormones.*

THs are also conjugated in the liver by UDP-glucuronosyltransferases (UGTs) forming glucuronide metabolites or via sulfotransferases (SULTs) forming sulphate metabolites (Figure 3) (Visser, 1988). TH-glucuronides are excreted into the bile and are partially reabsorbed due to deconjugation with β-glucuronidases in the intestine/microbiome entering an enterohepatic circulation. This reversible conversion of T4 and T3 is acting as a reservoir for these THs. The TH sulphates on the other hand are rapidly degraded by deiodinase enzymes to T2 sulphate or T1 sulphate, resulting in low levels in either plasma, bile and urine (M. Kester, 2001; Visser, 1988). Sulphation is marked as the primary step in the irreversible degradation of T4 and T3.

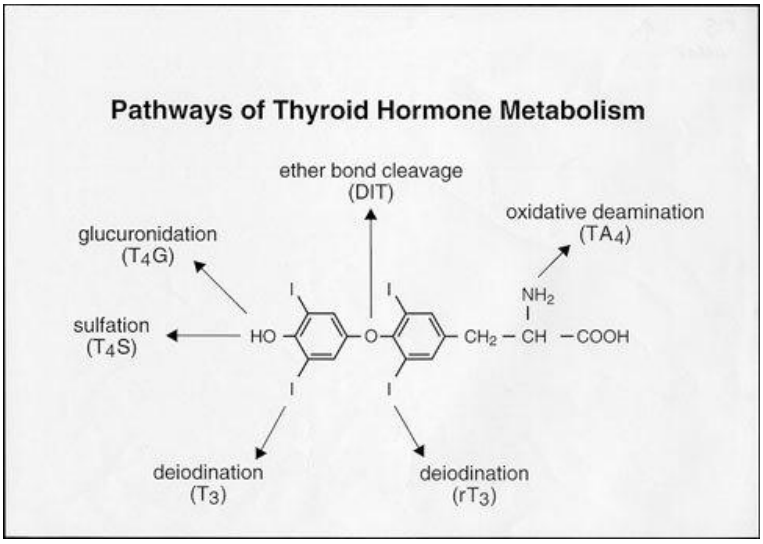

Figure 3 *Metabolic pathway of thyroid hormones.*

Disruption of the thyroid hormone homeostasis can happen by several mechanisms on the HPT axis and can be caused by exposure to chemicals (Claudio et al., 2000; Ghassabian & Trasande, 2018). Chemicals can influence the activity and gene expression of the TH receptor in the hypothalamus and pituitary gland but also the iodine uptake in the thyroid gland. One of the pathways to thyroid hormone disruption is the presence of chemicals having an effect on the gene expression of SULTs. When chemicals induce SULT gene expression more of the THs are sulphated and prone to undergo metabolism by deiodinase enzymes degrading them towards T2 sulphate and lower, thereby increasing the loss of TH, which eventually contributes to hypothyroidism (Visser, 1988). In contrast, inhibition of SULTs can result in less conversion of thyroid hormones leading to hyperthyroidism.

During gestation disruption of the thyroid hormone homeostasis in the mother may result in DNT as a disbalance in hormones will interfere with the development of the brain and central nervous system of the unborn child (Patel et al., 2011). As a consequence, these children have a higher chance of developing neurodevelopmental and neurological disorders such as learning disabilities, autism and ADHD (Bal-Price & Fritsche, 2018; Claudio et al., 2000). Even later in life these individuals may be more susceptible for developing Parkinson's or Alzheimer's disease.

1.1 Scope of the project

From animal studies (rats) it is evident that glucuronidation plays a major role in TH metabolism, where in humans sulphation seems to play an important role too (Baze et al., 2024). However, until today, little has been documented on validated methods to measure sulphation of THs. Therefore, in this research a method is proposed to investigate the use of primary human hepatocytes as an in vitro assay to measure sulphation of thyroid hormones. In addition, a set of possible positive controls is tested to induce the SULT expression as a first step towards validation.

1.2 Research tasks

This research was divided into three main tasks:

1. Developing an analytical method for liquid chromatography tandem mass spectrometry (LC-MS/MS) measurements.
2. Optimizing the sulphate metabolism assay for T3 and T4 using primary human hepatocytes.
3. Exploring the induction of SULT enzymes in primary human hepatocytes. Each of the tasks will be elaborated on further in the text, including.

2 Task 1. Development of an analytical method for LC-MS/MS measurements

2.1 Set up

An in-house method for the detection of T3, T4 and T4 glucuronide was available and used as a basis for the optimization of detection of T4-sulphate and T3-sulphate which is described below.

2.2 Materials and Methods

Analytical standards for T3, T4 and T4 glucuronide were already available at WFSR. Analytical standards for T4 sulphate and T3 sulphate were purchased from Santa Cruz and MedchemExpress, respectively. The instrument used was a Sciex 6500 LC triple quadrupole MS coupled to a Shimadzu UHPLC. Concentrations of 0.5 µM for all 5 compounds were prepared and injected to identify parent and product ions (see Table 1). The detection method was optimized.

2.3 Results and Discussion

The in-house method to detect T3, T4, and T4-glucuronide was using positive electron spray ionization (ESI). The substances to be added to the method, i.e. T3 sulphate and T4 sulphate however turned out to be ideally measured under basic conditions using negative ESI for optimal detection, sensitivity and peak stability. Fortunately, the TH compounds of the initial method could also be detected sufficiently under these conditions thereby providing a single analytical method (see Figure 4 for a chromatogram showing all 5 compounds). The details of the method are found below.

Method details LC

Eluent A	: milliQ Water + 10 mM ammonium carbonate
Eluent B	: 95% MeOH + 5% milliQ water + 10 mM ammonium carbonate
Column	: Acquity UPLC® BEH C18 column 1.7 µm
Injection volume	: 5 µL
Flow	: 0.4 mL/min
Runtime	: 15 minutes

Method details MS

ESI	: negative
Source temperature	: 400°C
Curtain gas	: 20
Ion source gas 1	: 50
Ion source gas 2	: 50
IonSpray voltage	: -4500 V

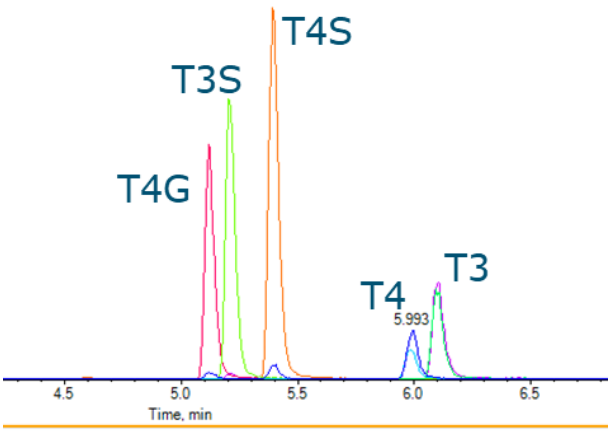

Figure 4 *LC-MS/MS chromatogram of T4, T4-glucuronide (T4G), T4-sulphate (T4S), T3 and T3-sulphate (T3S).*

Table 1 *Details for the analytical method of thyroid hormones T4 and T3 and their metabolites.*

Compound	Q1	Q3	CE	CXP	DP	Retention time (min)
T4	775.550	574.680	-52.000	-55.000	-125.000	5.99
	775.550	758.560	-38.000	-29.000	-125.000	
T3	649.600	632.700	-30.000	-27.000	-110.000	6.10
	649.600	448.800	-42.000	-49.000	-110.000	
T4G	951.500	775.600	-40.000	-33.000	-130.000	5.12
	951.500	574.600	-78.000	-51.000	-130.000	
T4S	855.500	775.600	-34.000	-39.000	-85.000	5.39
	855.500	574.600	-70.000	-55.000	-85.000	
T3S	729.600	649.700	-36.000	-27.000	-105.000	5.20
	729.600	632.700	-48.000	-25.000	-105.000	

2.4 Conclusions Task 1

An analytical method for the detection of thyroid hormones (T4 and T3) and their metabolites (T4-sulphate, T4-glucuronide and T3-sulphate) using LC-MS/MS has been successfully developed and can be used for the quantitative analyses expected in Task 2 and Task 3 of this research.

3 Task 2. Optimizing sulphate metabolism assay for T3 and T4

3.1 Set up

The goals within this task were:
1. To assess the metabolism of T4 and T3 at different incubation times.
2. To assess if the use of the deiodinase inhibitor propylthiouracil (PTU) increased the presence of sulphate metabolites.
3. To monitor the viability of the cells until day 8.

To this end, CryostaX® primary human hepatocytes (PHH) with 10 donors per vial were purchased from BioIVT (Lot: UDT) and used according to the suppliers' protocol.

3.2 Materials and Methods

A timeline for the culture of PHHs within this task is depicted in Figure 5. At day 0 the PHH were thawed and plated at 0.5 x 10^6 cells/mL onto collagen-coated 96 well plates containing 100 µL of cell suspension. According to the supplier, the cells can be used for experiments 18 hours after plating. On day 1, >18 hours after plating, metabolism studies were performed with T4 and T3 at a concentration of 1 µM each. Additionally, cells were exposed to 1, 10 and 100 µM PTU in the presence of 1 µM T4 and T3. Each condition was tested in triplicate and incubated for 0, 2, 4 and 24 hours. To verify the best sampling method, samples were collected by either taking the supernatant of the cells and directly add this to LC-MS/MS vials containing MeOH + 0.1 M NH3 (v/v 50:50) (sampling method 1) or by adding ice-cold MeOH + 0.1 M NH3 (50:50) to the wells to include cell lysis (sampling method 2). The plates with the lysed cells underwent one freeze-thaw cycle at -20°C whereafter they were centrifuged at 4000 rcf for 20 minutes at 4°C. Supernatant was collected in LC-MS/MS vials for further analysis with the method optimized in Task 1. The following control incubations were included: cells exposed to a solvent control (DMSO 0.1% in culture medium), medium only (without cells present), and T4 and T3 in culture medium without cells. The viability of the cells was monitored throughout the 8 days culture using the viability assay WST-1, which measures mitochondrial activity, on days 1 and 2, and LDH-leakage test, which measures cell membrane integrity, on days 2, 3, 5, 7 and 8. The readout for both assays was performed with a plate reader measuring absorbance. Cell morphology was inspected visually using phase-contrast microscopy.

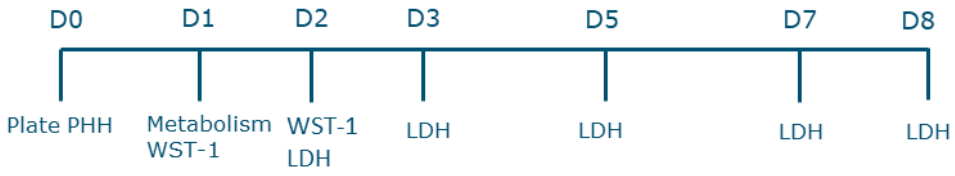

Figure 5 *Experimental time schedule using plated PHH for thyroid hormone metabolism and monitoring cells.*

3.3 Results and Discussion

Cell viability of CryostaX® PHH

Cell morphology remained stable throughout the 8 days in culture with only minor changes observed on later days (Figure 6a). Results from the WST-1 assay showed that viability remained around 100% on days 1 and 2, and following incubation with the test substances (Figure 6b). Results from the LDH leakage assay performed on days 2, 3, 5, 7 and 8 showed an average leakage of around 30%. There was an indication of minor cell deterioration observed over time.

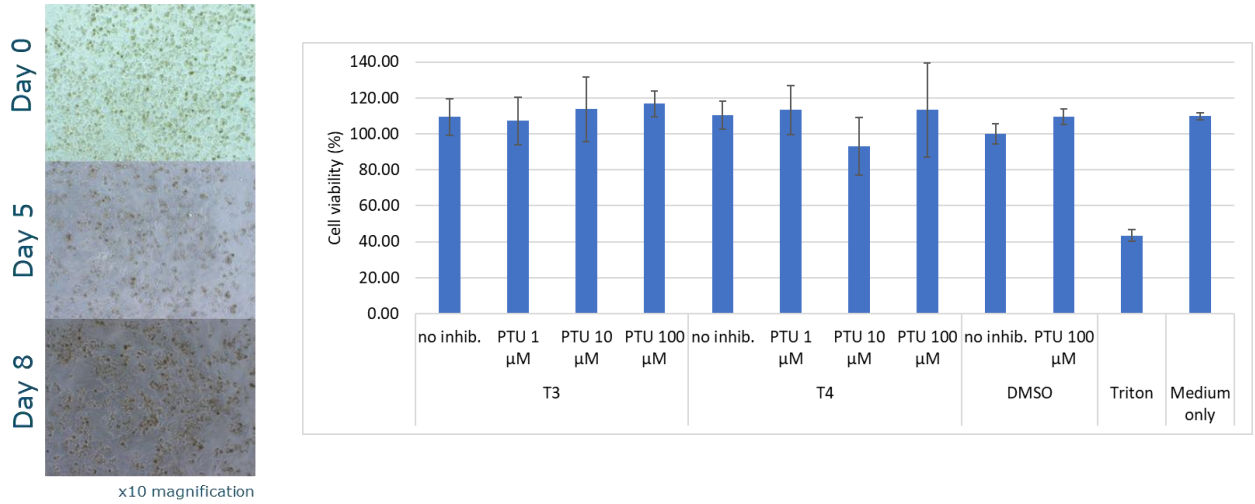

Figure 6 *Morphology and viability of the CryostaX® PHH throughout 8 days of culture. (a) Images of cells on days 0, 5, and 8. (b) Mitochondrial activity as determined by WST-1 assay on day 2 after the metabolism assay.*

Thyroid hormone metabolism

Formation of all three metabolites, i.e. T3S, T4S and T4G, was observed in incubations with CryostaX® PHH (Figure 7). Metabolite concentrations increased with increasing incubation time. Concentrations of T3-sulphate were highest, followed by T4-glucuronide, and T4-sulphate. Addition of PTU significantly ($p<0.05$) increased the concentration of T4 sulphate from a maximal concentration of 2.4 nM after 24h to 3 nM. This effect was observed at a PTU concentration of 1 µM, which did not increase further at higher concentrations of PTU. The formation of the other substances was not affected to a significant extent by addition of PTU.

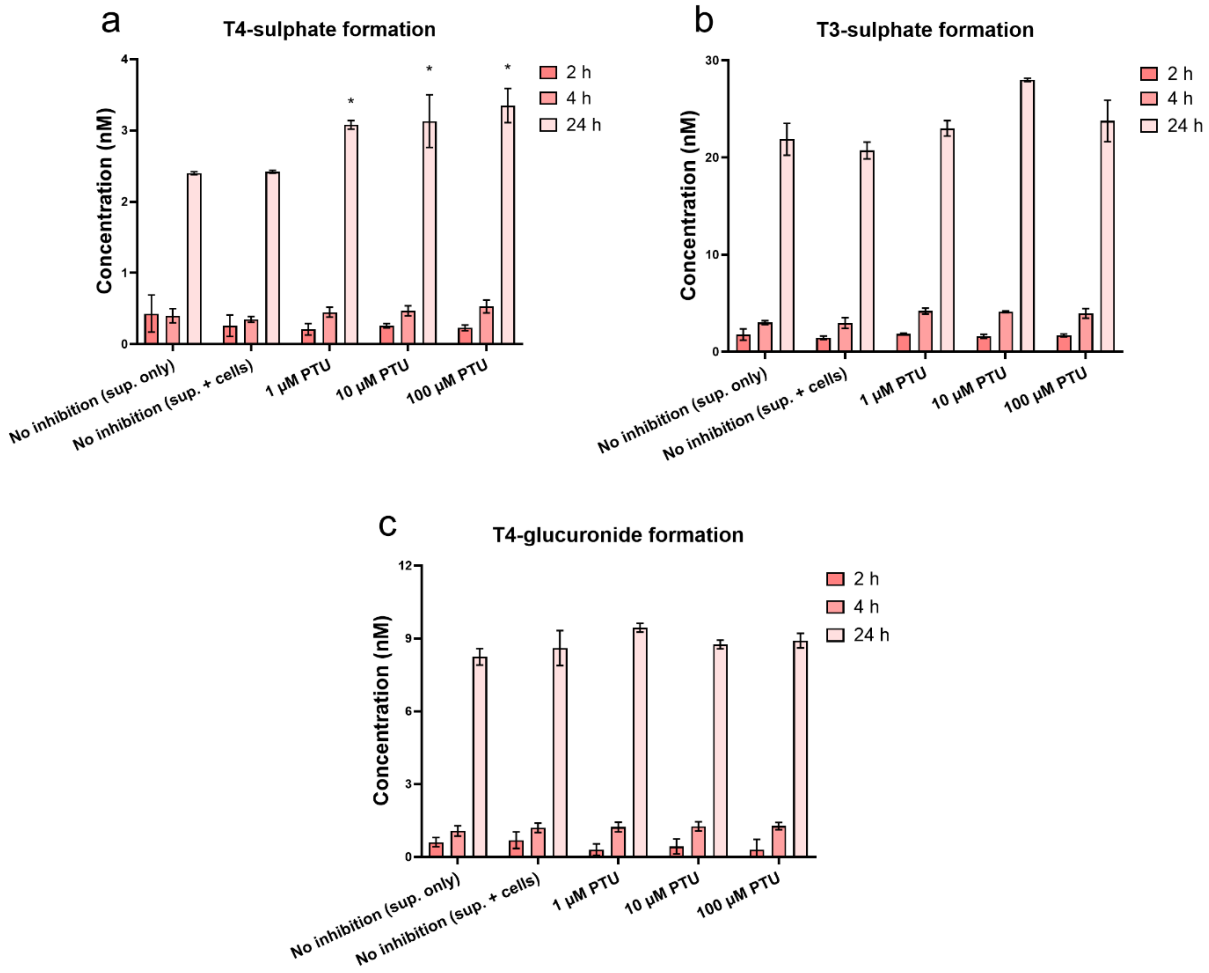

Figure 7 *Formation of (a) T4-sulphate, (b) T3-sulphate and (c) T4-glucuronide after incubating T4 and T3 with the CryostaX® PHH. The concentration used was 1 µM for both compounds and has been incubated for 2, 4 and 24 hours in the absence and presence of deiodinase inhibitor PTU tested at 1, 10 and 100 µM. Both supernatant alone (sup. only) and supernatant with cells (Sup. + Cells) were collected and compared. Each condition was tested in triplicate, * $p < 0.05$.*

3.4 Conclusions Task 2

Based on the activities performed in Task 2 the following conclusions can be drawn:

- The CryostaX® primary human hepatocytes are able to conjugate T4 to T4-sulphate and T4-glucuronide, and T3 to T3-sulphate.
- The presence of 1 µM PTU statistically inhibited deiodination thereby increasing the concentration of T4-sulphate. The presence of PTU did not alter the concentration of T3-sulphate.
- No differences were observed between the two sampling methods, therefore in the follow-up experiments sampling method 1 will be used (taking only the supernatant) allowing for an efficient workflow.
- For maximum formation of the sulphate and glucuronide metabolites, follow-up metabolism experiments will be carried out with a 24 hour incubation of thyroid hormone.
- The CryostaX® primary human hepatocytes show good morphology and viability throughout 8 days in culture.

4 Task 3. Induction studies for sulphate metabolism

This task aimed to identify chemicals that can induce SULT expression and activity and serve as positive control for the assay. To this end, a literature search was conducted and promising compounds were selected. The effect of these substances was tested in CryostaX® PHH and different conditions for inducing TH sulphation was compared. In this format, true positive controls should result in measurable elevation of T4-sulphate (via the increased expression of SULTs).

4.1 Literature search

The literature search focussed on the identification of
1. SULTs that are involved in the sulphation of T4 in liver,
2. nuclear receptors that are involved in regulating these SUTLs, and
3. compounds that induce gene/protein expression of SULTs.

In a study by Kester and coworkers (M. H. A. Kester et al., 1999) purified recombinant human sulfotransferases (SULT1A1, 1A3, 1B1 and 1E) were incubated with T4, T3, rT3, and T2. The results revealed that SULT1E1 can sulphate all four tested thyroid hormones. More specifically, SULT1E1 was identified as the key enzyme responsible for T4 sulphation, exhibiting at least a 20-fold higher sulphation rate than the other SULT isoforms. The order of sulphation for the remaining SULTs was identical following T2 > T3 > rT3> T4.

Riches et al., reviewed the expression and activity of five major human SULTs (Riches et al., 2009). Their studies showcased that the order of SULT expression in liver is as follows: SULT1A1 (53%) > SULT2A1 (27%) > SULT1B1 (14%) > SULT1E1 (6%) and SULT1A3 is found to be absent. Although the expression levels of SULT1E1 are relatively lower than other isoforms, its high activity for TH sulphation makes it a relevant SULT for the induction experiment because of its dominant role in the sulphation of T4.

The question arises what systems are affected by hormone disrupting toxins that act via the induction of SULTs. The literature review revealed that multiple nuclear receptors (NRs) are in volved in the regulation of SULT1E1. Several of them regulate the SULT1E1 gene by inducing its expression whereas others are reported to inhibit its expression. For the purpose of this study, the focus lied on identifying NRs that induce SULT1E1. According to literature, the main NRs that induce SULT1E1 are: peroxisome proliferator-activated receptor alpha (PPARα) and gamma (PPARγ), glucocorticoid receptor (GR), liver X receptor (LXR), and oestrogen receptor alpha (ERα). An additional search for compounds that evidently induce SULT1E1 via one of the abovementioned NRs resulted in a set of chemicals to include in this experimental step. As the number of compounds reported to substantially induce SULT1E1 is limited, one additional compound was included that is reported to induce (the 9 times more abundant) SULT1A1 via dopamine D1 receptor (D1R). While SULT1A1 has a lower activity towards T4 sulphation, the expression of this isoform is around 9 times higher in liver than that of SULT1E1. Table 2 sums up the proposed compounds for this experiment with associated NRs. As T4-glucuronide is present in the current analytical method and studied previously in our lab albeit in a different hepatic cell line (HepaRG) phenobarbital (PB) was added as positive control to induce UGT activity via the constitutive androstane receptor (CAR) (Sakakibara et al., 2016).

Table 2 *Potential inducers of SULT enzymes and known inducer of UGT enzymes.*

Compound	Nuclear receptor	Reference
SULTs		
Insulin-like growth factor 1 (IGF-1)	PPARα	(Li et al., 2013)
Dexamethasone (Dex)	GR	(Bian et al., 2007)
WY14643 (WY)	PPARα	(Li et al., 2013)
Dopamine (DA)	D1R	(Sidharthan et al., 2013)
UGTs		
Phenobarbital (PB) UGT-inducer	CAR	(Sakakibara et al., 2016)

4.2 Materials and Methods

The timeline of the experimental work within this task is depicted in Figure 8. The assumption made regarding the CryostaX® primary human hepatocytes was that metabolic activity was comparable on every day in culture until day 8. At day 0 the PHH (Lot: UDT) were thawed and plated onto collagen-coated 96 well plates. Starting on D1, the cells were exposed to the following inducers: dexamethasone (5 and 50 µM), WY14643 (5 and 50 µM) and dopamine (2.5 and 25 µM) all prepared from DMSO stocks and IGF-1 (10 and 100 ng/mL) prepared from a milliQ water stock. Phenobarbital (DMSO stock), the positive control for UGT activity, was added at a concentration of 1 mM that was reported in literature (Baze et al., 2024). All conditions were tested in triplicate. The cells were treated with the inducers for 2, 4, and 6 days to assess the optimal duration required for SULT enzyme induction. Medium with inducers was refreshed daily. After the induction, metabolism was tested by adding 1 µM of T4 with 1 µM PTU to inhibit deiodination of for 24 hours (see Task 2). Samples were collected by taking the supernatant of the cells and directly add this to LC-MS/MS vials containing MeOH + 0.1 M NH3 (v/v 50:50) for further analysis with the method optimized in Task 1. Control conditions included were: vehicle controls (DMSO and milliQ, both 0.1%), medium only and medium without cells.

Cell morphology was monitored visually, and viability assessed by performing LDH leakage and WST-1 assays. LDH leakage was monitored after induction and before metabolism of T4 on days 3, 5 and 7. WST-1 was performed after metabolism of T4 at days 4, 6 and 8.

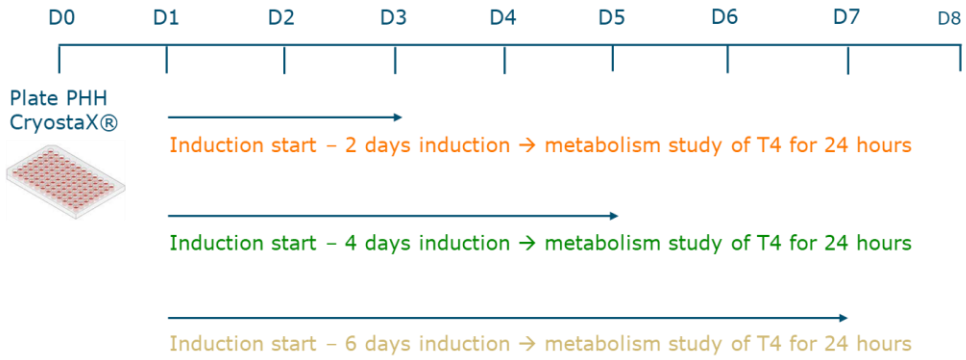

Figure 8 *Experimental time schedule for SULT induction in CryostaX® PHH combined with metabolism of T4.*

4.3 Results and Discussion

Results

Sulphate induction assay
Formation of T4-sulphate (Figure 9a) and T4-glucuronide (Figure 9b) was observed after 2 days (D3) and 4 days (D5) incubation with inducers, albeit in decreasing amounts. After 6 days of induction (D7), no metabolites could be detected in the samples. Incubations with the inducers did not significantly increase T4-sulphate formation; however, after 4 days induction, the presence of 25 µM dopamine increased the levels of both T4-sulphate and T4-glucuronide to some extent compared to the controls.

Cell viability data indicate that the leakage of LDH increases over time, indicating that the longer the cells are in culture, whether it is in the absence or presence of inducers, the more stress they seem to experience (supplementary data), which is not in line with what was found in the previous experiment. This could in part explain the decreasing sulphation activity observed. Mitochondrial activity measured in the WST-1 assay at days 4 and 6 showed that cell viability remained above the threshold for toxicity of 80% in all exposure conditions except for dexamethasone on day 6 (73%) (supplementary data). At day 8 mitochondrial activity was overall very low, indicating that the cells lost viability over time.

Glucuronide induction assay
As a positive control for T4-glucuronide formation the CryostaX® PHH were induced with PB at 1 mM. After 2 days of induction with PB, the concentration of T4-glucuronide was significantly ($p < 0.05$) increased over the solvent control by a factor of 1.6. After 4 days of induction, there was no increase in T4-glucuronide formation. This might be due to the long culture times, which appears to affect the vitality of the cells.

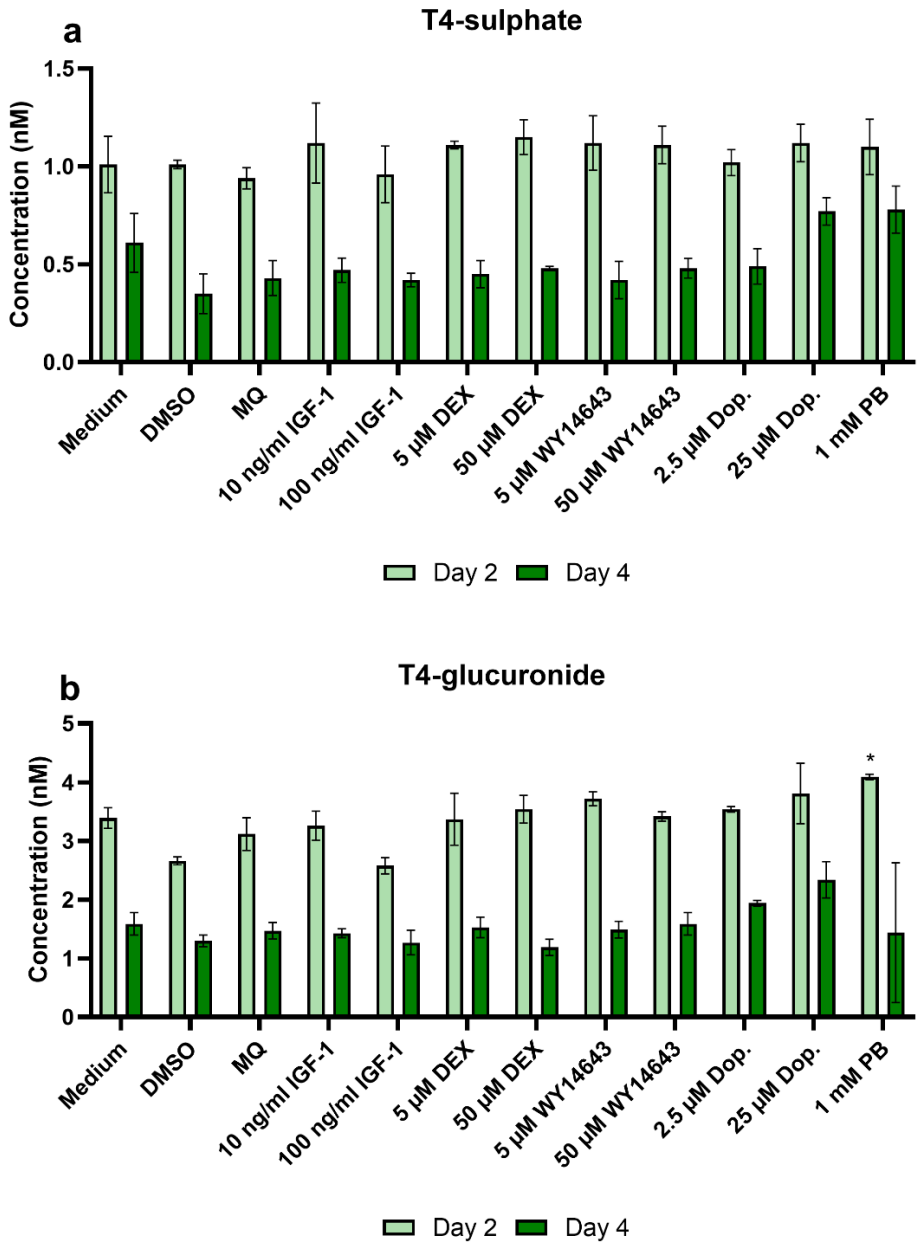

Figure 9 *Formation of (a) T4-sulphate and (b) T4-glucuronide by CryostaX® PHH after being induced with 10 and 100 ng/mL IGF-1, 5 and 50 µM dexamethasone (DEX), 5 and 50 µM WY14643, 2.5 and 25 µM dopamine to induce SULT expression and activity. Phenobarbital (PB) at 1 mM was included as a positive control for UGT induction. T4 was added at 1 µM for 24 hours after 2, 4 and 6 days induction. Each condition was tested in triplicate, * $p < 0.05$ compared to DMSO.*

Discussion

Under the optimized conditions in Task 2, metabolism experiments were performed with the primary hepatocytes after being induced with selected promising compounds to verify their potential as positive control for the induction of SULT enzymes. However, the observations in the sulphate induction assay appear to suggest that the compounds we selected are not active as such at all. Interestingly, the formation of T4-sulphate in the controls (and under induced conditions) is much lower after 4 days (D5) of induction than after 2 days of induction (D3) and is even absent after 6 days induction (D7) (data not shown). Comparing the T4-sulphate formation in the current Task with the T4-sulphate formation in Task 2 the concentrations observed are 3-fold lower. It therefore can be assumed that metabolic activity, which was tested on day 1 (D1) in Task 2, is diminishing over time.

The same is observed for the formation of T4-glucuronide, which was also 3-fold lower in the current Task compared to the formation in Task 2. Even though the formation is diminished, after 2 days of induction with the UGT positive control PB, an elevated concentration was observed, indicating the ability of the cells to increase formation of UGT enzymes after exposure. Results reported by Baze et al. (2024) revealed that 7 days induction would result in the highest formation of T4-glucuronide in PHH.

The assumption was that metabolic activity throughout the 8 days in culture would stay the same. However, from the results obtained in the current study it seems not the case. To overcome the loss of metabolic activity, the use of Matrigel is highly suggested as it mimics a natural liver microenvironment (Baze et al., 2024). Albeit that in light of working towards animal-free methods, the use of Matrigel is a contradictory suggestion so it would be most ideal to test available Matrigel alternatives in parallel.

With optimized conditions, whether that be with Matrigel or an alternative, more information shall be unraveled on the potential of the candidate compounds inducing SULT enzymes or whether to search for new candidates. This is also related to optimizing the duration of induction resulting in the optimum formation of SULT enzymes.
The discrepancy in cell viability measured in Task 2 and Task 3 lacks an unambiguous explanation. Handling of the cells was identical in both Tasks and the same lot number of cells was used. This however did not cover for the heterogenic nature of pooled cryopreserved primary hepatocytes.

4.4 Conclusions Task 3

Based on the activities performed in Task 3 the following conclusions can be drawn:

- Four compounds (IGF-1, dexamethasone, WY14643 and dopamine) have been selected from literature as candidates to induce SULT enzymes.
- No significant elevated sulphation activity (T4-sulphate) was observed after induction with the four selected compounds.
- Elevated glucuronidation activity (T4-glucuronide) was observed to a certain extent after a 2 day exposure to positive control phenobarbital. However, the effect was lost after 4 days exposure.
- The quality, cell viability and metabolic activity deteriorated during a culture period of 8 days.

5 Overall conclusions

The current study investigated the possibilities of using CryostaX® primary human hepatocytes as an *in vitro* model for metabolism of thyroid hormones. Specific attention was paid to sulphation activity of the hepatocytes and the selection of appropriate positive controls to induce sulfotransferases for elevated activity. The aim was to verify the potential of this *in vitro* model for future use as a validated testing method in the *in vitro* battery for testing developmental neurotoxicity. A brief conclusion of the 3 Tasks within this study is given below:

Task 1: An LC-MS/MS method for the detection and quantification of thyroid hormones thyroxine (T4), triiodothyronine (T3) and their metabolites T4 sulphate, T4 glucuronide and T3 sulphate was developed, successfully.

Task 2: CryostaX® primary human hepatocytes were evaluated for their use as metabolic test system for thyroid hormone sulphation and glucuronidation. The results following this evaluation indicated that the PHH are a promising test system for thyroid hormone metabolism.

Task 3: Four selected compounds for the induction of SULT enzymes have been tested in the PHH of which none elevated thyroid hormone sulphation. Cell culture optimization is required for proper testing of the SULT inducers.

6 Future research

Based on the obtained results in this study, further research is needed to move this assay forward as a candidate for validation. Due to the demonstrated high basal sulphation levels, this method still remains very promising. To further study the inducibility of sulphation, additional positive control substances need to be identified and tested, and optimizations of the culture conditions assessed. Steps in optimization are for example the use Matrigel and/or alternatives for Matrigel having a stabilizing effect on the metabolic activity of the PHHs, finding the optimal time for SULT induction, and additional effort to be put into measuring elevated SULT gene expression by PCR.

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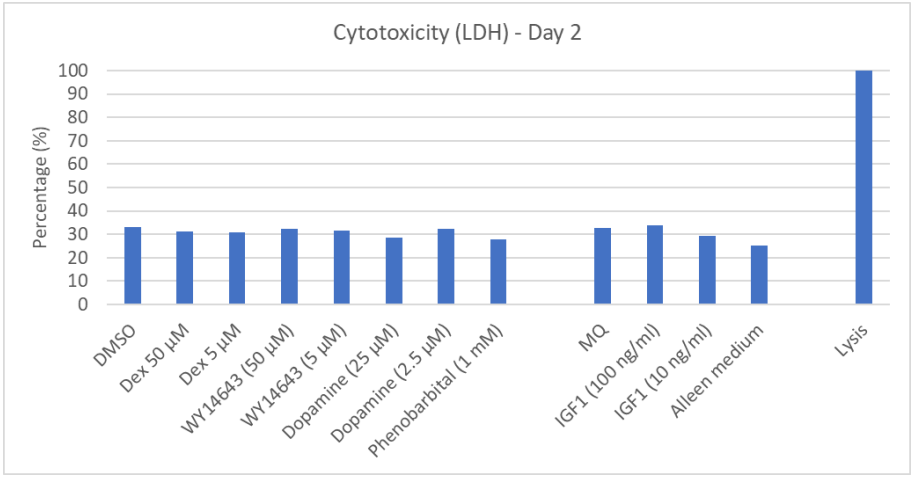
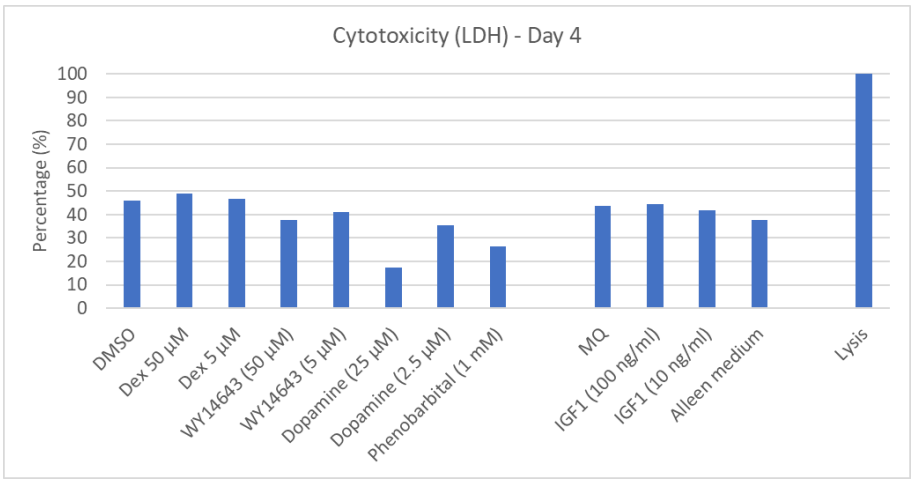
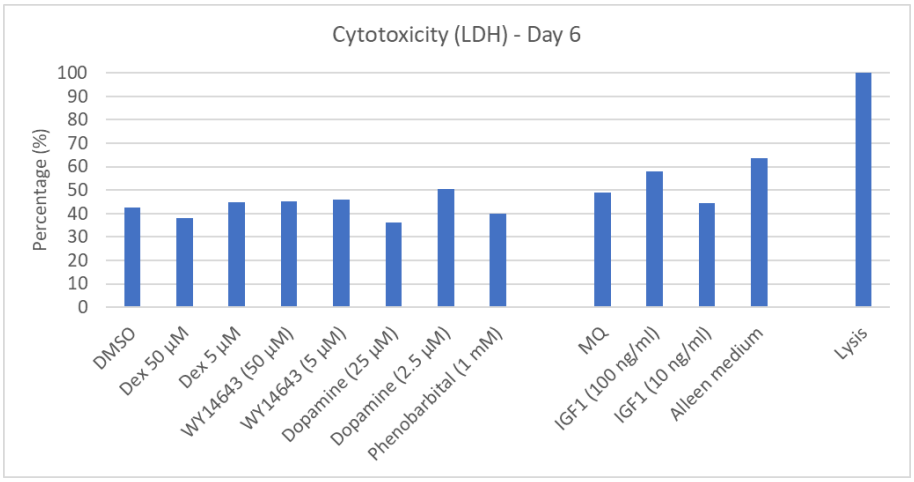
Supplementary data

Cell viability data corresponding to the work in Task 3.

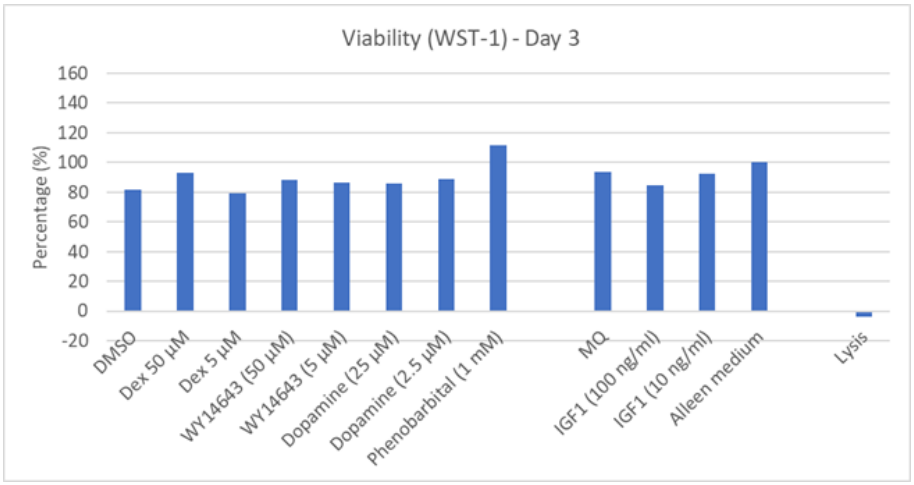
LDH assay indicating cytotoxicity on Day 2, 4 and 6. The higher the percentage (the more LDH the cells leak), the more stress the cells endure.

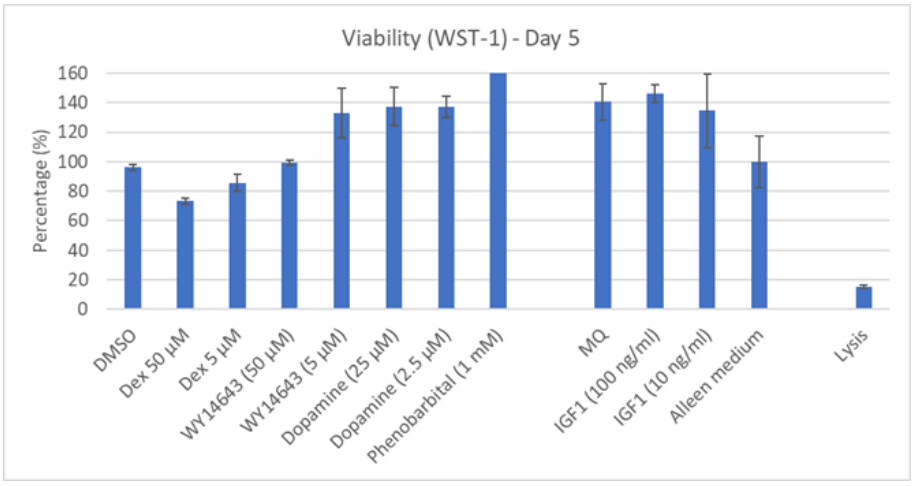


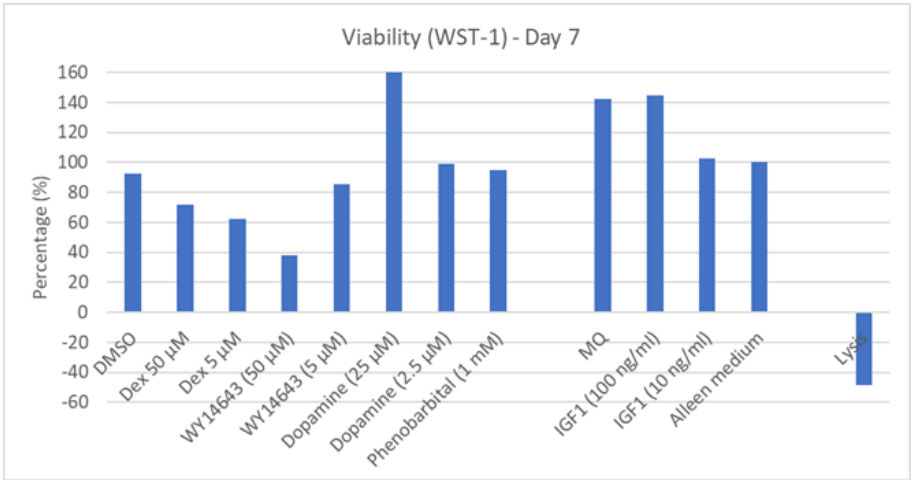




The WST-1 assay for measuring mitochondrial activity on Day 3, 5 and 7. The lower the percentage, the more loss of mitochondrial activity indicating a loss of viability in the cells.







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