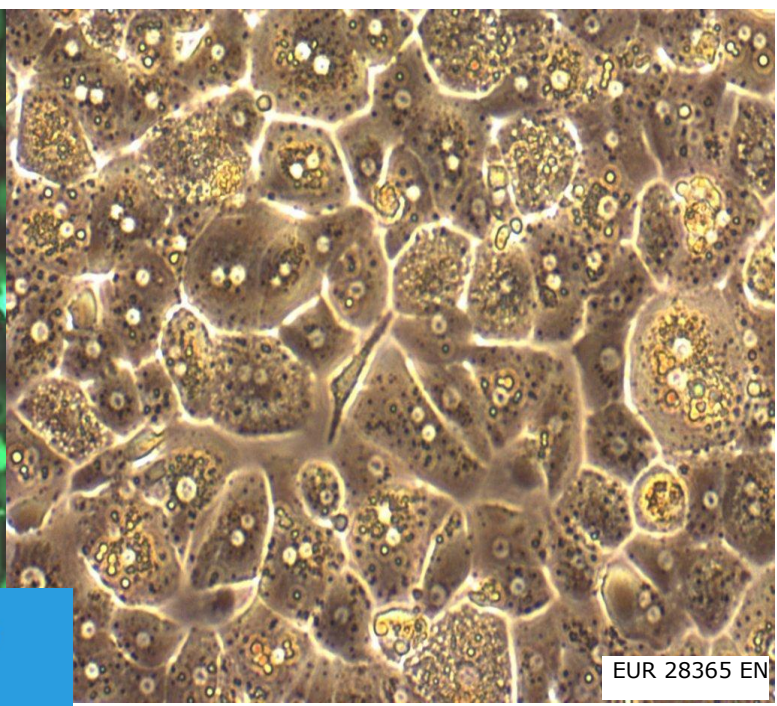
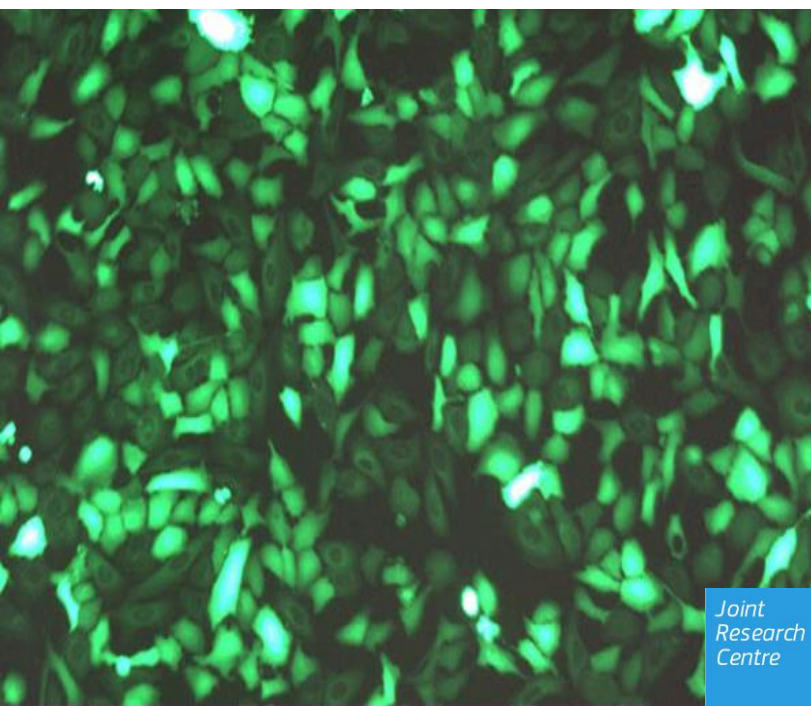


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Preliminary study to characterise the metabolic competence of *in vitro* cellular systems

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Abstract

The purpose of this report is to describe the experimental procedure used to perform a preliminary study to characterise the metabolic competence of *in vitro* cellular systems.

As a first study, the metabolic competence was assessed by monitoring the presence of four specific enzymes by using selected probe substrates. Each probe substrate can be metabolised by only one enzyme and therefore the formation over time of metabolites is indicative for the presence of a certain enzyme. The concentrations of each metabolite over-time were measured by using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS).

The method developed to monitor the presence of the four enzymes was then used to compare the metabolic competence between two cellular systems: HepaRG (human liver cells known to be metabolically competent) and AR-CALUX (human osteoblastic osteosarcoma U2-OS cells having low or none metabolic activity).

The results and experience achieved will serve to ultimately support the planned work of Directorate F3 focused on developing an EURL ECVAM recommendation, which will be followed by an OECD Guidance Document, with the objective to characterise and describe *in vitro* hepatic metabolic clearance methods in order to facilitate their regulatory uptake and use to support chemical risk assessment.

1 Introduction

Modern toxicology is relying more and more on the use of alternative methods to animal testing in order to predict if a chemical can be potentially toxic to humans.

The traditional approach, which is still highly used to support regulatory chemical risk assessment, is the administration of different doses of a chemical to animals with the scope to evaluate if the chemical causes adverse effects.

For a variety of reasons, from ethical considerations on animal welfare to the need generate more accurate information on chemical's toxicity in less time and with less resources, several *in vitro* cell methods have been developed or are under development.

In vitro methods are based on a biological system which can be either a cellular system (e.g a specific cell type representing a targeted human organ as the liver, kidney, lung, etc.) or a sub-cellular system (e.g enzymes isolated from a specific cell or cellular membranes, etc.).

The general workflow to predict chemical's toxicity by using *in vitro* tests implies exposing cells (or sub-cellular fractions) to several concentrations of a certain chemical to then measure biological endpoints that are informative of the chemical toxicity.

In other words, the objective is to evaluate what the chemical does to the biological system and this process is also called dynamics.

However also the cellular system does something to the chemical and this process is called kinetics. In a cellular system the kinetics process involves the direct excretion of the chemical from the cell, or its metabolic biotransformation to other chemicals species (metabolites) which can facilitate the excretion from the cell. The cellular metabolism (or *in vitro* metabolic clearance) is in many cases the main driving process of *in vitro* kinetics to determine the concentration-time profile of a chemical in cellular system.

To summarise, the interaction between an *in vitro* cellular system and a chemical implies both kinetics and dynamics processes which happen in parallel and their combination can influence the manifestation of a toxic effect caused by exposure to a chemical.

Therefore, the kinetics, and particularly the *in vitro* metabolism, plays an important role in the manifestation of potential toxicity since it influences the amount of a chemical over time that can interact with the biological system and eventually causes toxicity.

Prediction of *in vivo* toxicity by using *in vitro* assays is promising but there are still limitations particularly to predict complex toxicological endpoints.

There are several explanations why *in vitro* data fail to predict certain *in vivo* endpoints and one of these is the difference in metabolic clearance,

and in general of metabolic machinery, between *in vitro*- and *in vivo*-assays.

A different metabolic pathway of the *in vitro* assay might then hamper the relevance of the *in vitro* data when they are used to predict the *in vivo* counterpart.

For example, a chemical might be considered toxic based on the *in vitro* data even if *in vivo* results show no toxicity. One possible explanation is that the *in vitro* system used for toxicity testing is not metabolically competent and therefore the chemical accumulates within the cell causing toxicity. This does not happen in *in vivo* where the chemical is metabolised to a non-toxic metabolite which is then excreted from the cells without causing toxicity.

Therefore the metabolic characterisation of an *in vitro* test system, to evaluate the level of similarity of the metabolic machinery with the *in vivo* counterpart, can serve to select the most suitable *in vitro* system and to better assess the relevance of *in vitro* data when used to predict chemical toxicity.

The metabolic machinery is comprised of several enzymes also called biotransformation enzymes and its characterisation should monitor the presence of each enzyme or of those of interest for a specific purpose. Biotransformation is usually divided into two main phases, phase I and phase II. Phase I is usually oxidative (e.g. hydrolysis, although reductive metabolism and non-redox reactions of functionalization can also occur), and has a major protective function in making lipophilic molecules more polar and therefore facilitating their excretion. In phase II, which is often called as detoxification phase, the chemicals oxidised in phase I are subsequently conjugated with highly polar molecules, such as glucuronic acid, to further facilitate their excretion.

The metabolic characterisation, e.g. the identification of the presence of Phase I and Phase II biotransformation enzymes in a biological cell, tissue or subcellular fraction, is generally performed by incubating a probe substrate which can be metabolised by only one specific enzyme and measuring the metabolite formed. The rate of metabolite formation, expressed as pmol/min/mg protein is the expression of the activity of each specific enzyme. The characterisation of multiple enzymes requires the use of multiple probe substrates.

For metabolic characterisation, the cells, tissues or subcellular fractions are exposed with one or multiple probe substrates at different incubation time-points and the known metabolites are usually measured using dedicated analytical methods and instrumentation such as liquid chromatography-tandem mass spectrometry (LC-MS/MS).

This report describes a preliminary work performed at EURL ECVAM to characterise the metabolic competence of *in vitro* cellular systems. EURL ECVAM is interested to better characterise *in vitro* cellular systems as a

mean to improve their evaluation in the context of how they can be used to support chemical risk assessment. Therefore, the assessment of metabolic competence is one parameter to be considered when characterising the properties of *in vitro* cellular systems.

In this study, the presence of the following four biotransformation enzymes was characterised by using specific probe substrates selected from previous literature studies [1]:

- Cytochrome P450 2E1 (CYP2E1)
- Monoamine oxidase (MAO)
- Uridine 5'-diphospho-N-acetylgalactosamine glycosyltransferase (UGT)
- N-Acetyltransferase (NAT)

These enzymes were selected since they have a broad coverage of both phase I and phase II metabolism.

The method was implemented, and used to characterise the metabolic competence of the following two *in vitro* cellular systems: AR-CALUX cells and CryoHepaRG.

HepaRG cells, which are human liver cells of cancer origin, are known to be metabolically competent and they have a similar metabolic machinery to the one of primary human hepatocytes. Whereas, AR-CALUX are human osteoblastic osteosarcoma U2-OS cells having low or no metabolic activity.

Therefore this study was performed, as proof of concept, to compare the metabolic machinery of two cell lines known to represent to different situations.

2 Materials and Methods

2.1 General experimental layout

In this study, the metabolic competence of two cell lines, CryoHepaRG and AR-CALUX, was evaluated and compared by monitoring the presence of the following four enzymes: CYP2E1, MAO, UGT and NAT.

Each cell line was exposed for 1 h to each individual probe substrate, at two different concentrations in triplicates, in order to start the incubation.

Each probe substrate is specifically metabolised by only one of the four above enzymes to be monitored. Therefore, the presence of the selected enzymes is evaluated by measuring the specific metabolites, eventually formed, from each of the probe substrates.

At the end of the incubation period (1 h), the selected metabolites eventually formed are measured by LC-MS/MS analysis.

Figure 1 represents the experimental layout.

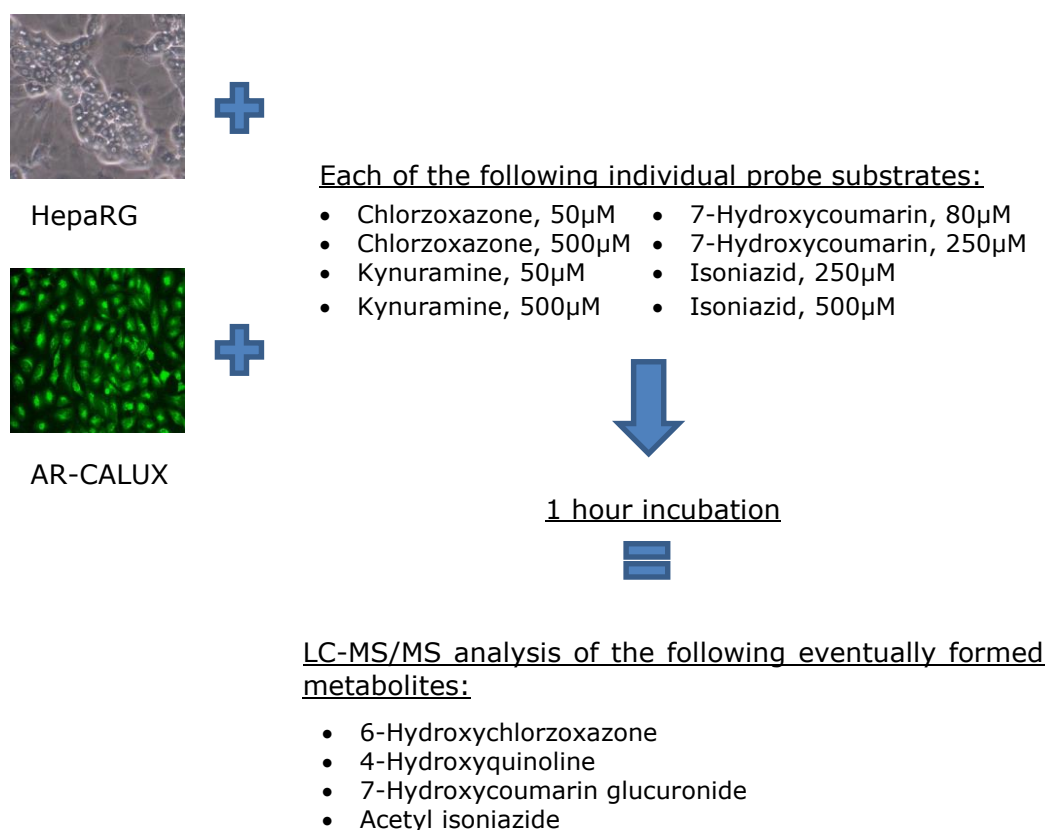


Figure 1

2.2 Materials

2.2.1 Chemicals:

- Chlorzoxazone (CAS No. 95-25-0, Sigma-Aldrich)
- 6-Hydroxychlorzoxazone (CAS No. 1750-45-4, Carbosynth)
- Kynuramine (CAS No. 363-36-0, Chemos)
- 4-Hydroxyquinoline (CAS No. 611-36-9, Sigma-Aldrich)
- 7-Hydroxycoumarin (CAS No. 93-35-6, Sigma-Aldrich)
- 7-hydroxycoumarin glucuronide sodium salt (CAS No. 66695-14-5, Carbosynth)
- Isoniazid (CAS No. 54-8-3, Sigma-Aldrich)
- Acetyl isoniazide (CAS No. 1078-38-2, Carbosynth)
- 5,5-Diethyl-1,3-diphenyl-2-iminobarbituric acid, DDIBA (CAS No. 200116-26-3, Sigma-Aldrich) used as internal standard for LC-MS analysis.

2.2.2 Cells, cell media and additives:

CryoHepaRG cells (Biopredic)

- Williams' Medium E
- 200 mM L-glutamine
- HepaRG® Thaw, Seed and General Purpose Supplement (contains serum, Invitrogen, HPRG670)
- HepaRG® Induction Supplement (with no serum, Invitrogen, HPRG650)
- 1 M HEPES buffer

AR-CALUX cells (BioDetection Systems, BDS)

- DMEM supplemented with F12 with phenol red
- DMEM supplemented with F12 without phenol red
- 7.5% Foetal Calf Serum, FCS
- 5% Dextran-Coated Charcoal treated FCS
- 1% non-essential amino acids
- 10 Units/ml Penicillin
- 10 µg/ml Streptomycin
- 0.02% Geneticin

2.2.3 Plastic-ware and disposable:

For CryoHepaRG cells:

- 1.5 ml, 15 ml und 50 ml centrifugation tubes, conically shaped, polypropylene, sterile;
- 96-well plates with lid, uncoated, polypropylene;
- 96-well plates coated with collagen I, qualified for seeding and culture of CryoHepaRG® (e.g. Biopredic International, PLA136)

For AR-CALUX cells:

- Clean or sterile glass tubes or vials;
- Sterile pipettes, 1, 2, 5, 10 and 25 ml;
- Sterile tips, 10, 100, 200, 1000 and 5000 µl;
- 96-well plates with lid, uncoated, polypropylene;
- Cell culture flasks 75 cm²;

2.2.4 Technical Equipment:

- 37°C cell culture incubator with a 5±1% CO₂ atmosphere and saturated humidity
- Water bath able to maintain a temperature of 37°C
- Centrifuge

2.2.5 Instrument used for chemical analysis:

HPLC 1100 G1312A (Agilent) coupled with API 4000 Triple Quadrupole (Perkin-Elmer Sciex Instruments)

2.3 Enzymes monitored and corresponding probe substrates

Table 1

Enzyme	Phase of metabolism	Probe Substrate added to cells	Metabolite formed
CYP2E1	I	Chlorzoxazone	6-Hydroxychlorzoxazone
Mono Amine Oxidase	I	Kynuramine	4-Hydroxyquinoline
Uridine 5'-diphospho-N-acetylgalactosamine glycosyltransferase	II	7-Hydroxycoumarin	7-Hydroxycoumarin glucuronide

N-Acetyltransferase	II	Isoniazid	Acetyl isoniazide
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2.4 Material Preparation

2.4.1 HepaRG:

2.4.1.1 Preparation of HepaRG basal medium:

- 1) Transfer 99 mL Williams's Medium E in a plastic container
- 2) Add 1 mL of L-Glutamine 200 mM
- 3) Shake thoroughly and label as "HepaRG basal medium"
- 4) Assign an expiry date of 1 month

2.4.1.2 Preparation of HepaRG Thaw, Seed and General Purpose Medium (contains serum):

- 1) HepaRG® Thaw, Seed and General Purpose Supplement (contains serum) is thawed by placing the vial (12.5 mL) into a 37°C water bath. Then the volume is transferred into a new plastic bottle
- 2) Add 100 of "HepaRG basal medium" (see section 2.4.1.1)
- 3) Shake thoroughly and label as "HepaRG Thaw, Seed and General Purpose Medium"
- 4) Assign an expiry date of 1 month

2.4.1.3 HepaRG Serum-Free Induction Medium:

- 1) HepaRG Induction Supplement (with no serum) is thawed by placing the vial (0.6 mL) into a 37°C water bath. Then the volume is transferred into a new plastic bottle
- 2) Add 100 of "HepaRG basal medium" (see section 2.4.1.1)
- 3) Shake thoroughly and label as "HepaRG® Serum-Free Induction Medium"
- 4) Assign an expiry date of 1 month

2.4.1.4 Incubation Medium:

- 1) Take a bottle of 500 mL Williams's Medium E and use it for the following steps:
- 2) Add 5 mL of L-Glutamine 200 mM
- 3) Add 12.5 mL of HEPES
- 4) Shake thoroughly and label as "Incubation medium"

5) Assign an expiry date of 1 month

2.4.1.5 Thawing and culturing of CyroHepaRG cells:

Note 1: 1 vial contains about 8 million of cryopreserved HepaRG. Since, for one experiment (where all the 4 probe substrates are added to cells at two concentrations each in triplicates), each well contains 72000 cells, 1 vial is sufficient to perform an experiment following the layout shown in following **Figure 2**.

Note 2: the thawing must be performed on a Friday and the seeded cells are allowed to settle over the weekend in 96-wells plates

The thawing and culturing of CyroHepaRG cells have been previously described [2], below are the critical steps of the protocol

1. Pre-warm HepaRG Thaw, Seed and General Purpose Medium (see section 2.4.1.2) to 37°C for about 10-20 min
2. Transfer 9 ml of pre-warmed HepaRG Thaw, Seed and General Purpose Medium to a sterile 50 mL polystyrene tube
3. Carefully remove the vial from the cryostorage
4. Immerse the vial into a 37°C water bath. Shake it gently for about 1.5 minutes until the ice is detached from the plastic. It is very important that the vial is not submerged completely to avoid water penetration into the cap. Furthermore, small ice crystals should remain when the vial is removed from the water bath
5. Transfer the vial to the laminar flow hood and disinfect it with an absorbent paper containing isopropanol or ethanol
6. From the vial, transfer all CyroHepaRG cells suspension into the pre-warmed 50 mL polystyrene tube containing 9 mL of HepaRG® Thaw, Seed and General Purpose Medium
7. From the 50 mL polystyrene tube, use 1 mL of solution to rinse the vial once. Then, transfer back 1 mL into the 50 mL polystyrene tube
8. Re-suspend the hepatocytes suspension by gently inverting the 50 mL polystyrene tube 3 times
9. Centrifuge the 50 mL polystyrene tube at 350 g at room temperature for 2 minutes
10. Discard the supernatant by aspirating using a vacuum pump
11. Add 5 mL of pre-warmed HepaRG Thaw, Seed and General Purpose Medium to the 50 mL polystyrene tube. Then, using a pipette, pipette gently for loosening the pellet
12. Determine the total cell count and the percentage of viable cells using the Trypan Blue exclusion method
13. After determination of the cell concentration (expressed as cells/mL), add additional pre-warmed Cell Medium to obtained the desired concentration of cells (**for the incubation assay**)

72000 cells in 100 µL are needed. Therefore the desired final concentration is 720000 cells in 1 mL of Cell Medium)

Acceptance criteria to use hepatocytes for incubation assay

- A minimum cell viability , by using the Trypan Blue method, of 80% after thawing must be obtained.
14. After reconstitution at the desired concentration (720k/mL), the cells are transferred to a 96-well plate coated with collagen I, suitable for seeding and culture of CryoHepaRG® (e.g. Biopredic International, PLA136)" for seeding. 100 µl (containing 72000 cells) are transferred to each well (see following **Figure 2** for the plate layout)
 15. 6 h from after the plating, cells are already attached and the medium is replaced with fresh pre-warmed one. Then, the cells are left in the incubator at 37°C over the weekend
 16. On Monday, the medium is replaced with HepaRG® Serum-Free Induction Medium (see section 2.4.1.3), renewed after 24 h. The cells are left in this medium at 37°C for 48h and then they are ready to be treated

2.4.2 AR-CALUX:

2.4.2.1 Preparation of Growth medium:

- 1) Place a vial of 41 ml of 7.5% Foetal Calf Serum into a 37°C water bath for thawing
- 2) Open a 500 mL bottle of DMEM/F12 with phenol red in a laminar flow cabinet
- 3) Add 5.5 ml of non-essential amino acids (MEM 100x) to get 1 %
- 4) Add the tube containing the 41 ml of FCS thawed (point 1)
- 5) Add 1 ml penicillin-streptomycin solution
- 6) Add 2.2 ml Geneticin
- 7) Shake thoroughly and label as "Growth Medium"
- 8) Assign an expiry date of 1 month

2.4.2.2 Preparation of Assay medium:

- 1) Place a vial of 26.6 ml of 5% Dextran-Coated Charcoal treated FCS into a 37°C water bath for thawing
- 2) Open a 500 mL bottle of DMEM/F12 without phenol red in a laminar flow cabinet
- 3) Add 5.5 ml of non-essential amino acids (MEM 100x) to get 1 %
- 4) Add the tube containing the 26.6 ml of DCC-FCS (point 1)
- 5) Add 1 ml penicillin-streptomycin solution

- 6) Shake thoroughly and label as "Assay Medium"
- 7) Assign an expiry date of 1 month

2.4.2.3 Thawing and culturing of AR-CALUX cells:

Note 1: The AR-CALUX cells were thawed two weeks before the treatment because, according to the acceptance criteria, the cells need to have at least 2 passages (two per week) in order to then be used for the experiment. For this experiment the cells were grown for 4 passages.

Note 2: On Tuesday morning of the week of the experiment (in order to use AR-CALUX cells with HepaRG ones in the same experiment), the cells were re-suspended in assay medium and seeded at 10000 cells/well (100 μ l from a 100000 cells/ml cell suspension).

1. From the refrigerator (at 4°C), take the Growth medium (see section 2.4.2.1) and pipet 15 ml of it a sterile 50 mL polystyrene tube. Then put the 50 mL polystyrene tube back in the refrigerator
2. Pre-warm the remaining growth medium in a water bath at 37°C.
3. Carefully remove the vial from the cryostorage
4. Immerse the vial into a 37°C water bath. Shake it gently for about 1.5 minutes until the ice is detached from the plastic. It is very important that the vial is not submerged completely to avoid water penetration into the cap. Furthermore, small ice crystals should remain when the vial is removed from the water bath
5. Transfer the vial to the laminar flow hood and disinfect it with an absorbent paper containing isopropanol or ethanol
6. Retrieve the 50 mL polystyrene tube containing 15 mL of Growth Medium which was kept in the refrigerator at 4°C (point 1) and , transfer into it all AR-CALUX cells suspension from the vial
7. From the 50 mL polystyrene tube, use 1 mL of solution to rinse the vial once. Then, transfer back 1 mL into the 50 mL polystyrene tube
8. Re-suspend the hepatocytes suspension by gently inverting the 50 mL polystyrene tube 3 times
9. Centrifuge the 50 mL polystyrene tube at 250 g at room temperature for 5 minutes
10. Discard the supernatant by aspirating using a vacuum pump
11. Add 10 mL of pre-warmed Growth Medium to the 50 mL polystyrene tube. Then, using a pipette, pipette gently for loosening the pellet
12. Transfer the 10 mL with the cells re-suspended into a culture flask 75 cm² (passage number 0)
13. Place the culture flask in the incubator at 37°C.

14. When the cells reach 85-95% confluence (it takes about 2 days), they are trypsinased and then transferred into a new flask (passage 1). The seeding density for sub-culturing cells in a 75 cm² flask should be of 110000 cells/mL (1100000 cells per flask of 10 mL) to reach the 85-95% confluence in 3 days for the next subculturing day, or it should be of 70000 cells/mL (700000 cells per flask of 10 mL) to reach the 85-95% confluence in 4 days for the next subculturing day. This is to be taking into account to avoid subculturing the cells on weekends
15. Repeat steps 13 and 14 to reach passage 4
16. When cells have reached passage 3 or 4 (therefore they can be used for the experiment), remove the growth medium
17. Add in the flask 10 ml of Assay medium (see section 2.4.2.2) and transfer to a sterile 50 ml tube.
18. Determine the total cell count and the percentage of viable cells using the Trypan Blue exclusion method
18. After determination of the cell concentration (expressed as cells/mL), add additional Assay Medium to obtained the desired final concentration of 100000 cells in 1 mL
19. After reconstitution at the desired concentration, the cells are transferred to "96-well plates with lid, uncoated, polypropylene" for seeding. 100 µl (containing 10000 cells) are transferred to each well (see following **Figure 2** for the plate layout)
20. The cells are left in the incubator at 37°C for 24 h prior the experiment

2.4.3 Preparation of working concentrations of probe substrates:

Each probe substrate (see **Table 1**), was prepared at 10 mM in methanol (stock concentration). Then each stock was used to prepare the working concentrations summarised in **Table 2**. The working concentrations used to expose HepaRG were made in incubation medium (refer to section 2.4.1.4) while working concentrations used to expose AR-CALUX were made in assay medium (refer to section 2.4.2.2)

Table 2

CYP2E1 (Phase I)	MAO (Phase I)	UGT (Phase II)	NAT (Phase II)
Chlorzoxazone	Kynuramine	7-Hydroxycoumarin	Isoniazid
conc 1 = 50µM	conc 1 = 50 µM	conc 1 = 80µM	conc 1 = 250µM
conc 2 = 500µM	conc 2 = 500µM	conc 2 = 250µM	conc 2 = 500µM

2.5 Method

2.5.1 Study design

After the culturing of HepaRG and AR-CALUX cells (as described in section 2.4.1.5 and 2.4.2.3), the assay consists of exposing each cell line to each of the four probe substrates at two concentrations in triplicates for 60 min.

At the end of the 60 min exposure, the incubation reaction was stopped by the addition of a stop solution (acetonitrile containing 5-Diethyl-1,3-diphenyl-2-iminobarbituric acid, DDIBA used as Internal Standard) and the samples were analysed for the specific metabolites shown in **Table 1** by means of LC/MS/MS.

The 96-well plate layout shown in **Figure 2** is then used to expose HepaRG or AR-CALUX (one plate/cell type).

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	Cells + Chlorzoxazone 50µM	Cells + Kynuramine 50µM	Cells + 7-hydroxycoumarin 80µM	Cells + Isoniazid 250µM				Negative Control Cells with no chemical)				
C	Cells + Chlorzoxazone 50µM	Cells + Kynuramine 50µM	Cells + 7-hydroxycoumarin 80µM	Cells + Isoniazid 250µM				Negative Control Cells with no chemical)				
D	Cells + Chlorzoxazone 50µM	Cells + Kynuramine 50µM	Cells + 7-hydroxycoumarin 80µM	Cells + Isoniazid 250µM				Negative Control Cells with no chemical)				
E	Cells + Chlorzoxazone 500µM	Cells + Kynuramine 500µM	Cells + 7-hydroxycoumarin 250µM	Cells + Isoniazid 500µM								
F	Cells + Chlorzoxazone 500µM	Cells + Kynuramine 500µM	Cells + 7-hydroxycoumarin 250µM	Cells + Isoniazid 500µM								
G	Cells + Chlorzoxazone 500µM	Cells + Kynuramine 500µM	Cells + 7-hydroxycoumarin 250µM	Cells + Isoniazid 500µM								
H												

Figure 2

2.5.2 Incubation assay

1. Pre-warm the working concentrations of probe substrates (previously prepared (see section 2.4.3) at 37°C.
2. Remove from the incubator at 37 °C the two 96-well plates (one for HepaRG and one for AR-CALUX, see section 2.4.1.5 and section 2.4.2.3)
3. From each plate, remove the cell medium and wash each well twice with 100 µL of Incubation Medium (section 2.4.1.4) for HepaRG and Assay medium (section 2.4.2.2) for AR-CALUX as shown in Figure 2
4. After the washing, to each well, as shown in Figure 2 (except to the wells labelled as negative control), add 50 µL of the corresponding probe substrate (the probe substrates added to HepaRGs were prepared in Incubation medium while those for AR-CALUX in Assay medium, see section 2.4.3)
5. Incubate the 96-well plates at 37°C for 60 min
6. At the end of exposure time, transfer from each well 40 µL of cell medium (with no cells since they are still attached) to the corresponding well of a new 96-well plate and immediately, to each well, add 40 µL of stop solution (containing acetonitrile containing ,5-Diethyl-1,3-diphenyl-2-iminobarbituric acid, DDIBA Internal Standard)
7. Centrifuge the plates for 10 min at 2,200 g
8. From each well (containing 80 µL volume) transfer 30 µl volume into a new 96-well plate (correspondingly labelled as "LC/MS analysis plate") and diluted with 70 µl H₂O. This plate is covered with solvent-resistant aluminium foil and stored at -20°C until LC-MS analysis.

2.5.2.1 Protein determination

The two 96-well plates (one for HepaRG and one for AR-CALUX) with the cells seeded were then used for the protein determination.

For the protein determination the Micro BCA™ Protein Assay Reagent Kit was used. It is a detergent-compatible bicinchonic acid (BCA) formulation for the colorimetric detection and quantitation of total protein. The method utilises BCA as the detection reagent for Cu⁺¹ which is formed when Cu⁺² is reduced by protein in an alkaline environment. A purple colored reaction product is formed by the chelation of two molecules of BCA with one cuprous ion (Cu⁺¹). This water soluble complex exhibits a strong absorbance at 562nm that is linear with increasing protein concentration.

The BCA assay primarily relies on two reactions.

First, the peptide bonds in protein reduce Cu^{2+} ions from the copper (II) sulfate to Cu^+ (a temperature dependent reaction). The amount of Cu^{2+} reduced is proportional to the amount of protein present in the solution. Next, two molecules of bicinchoninic acid chelate with each Cu^+ ion, forming a purple-colored complex that strongly absorbs light at a wavelength of 562 nm.

The bicinchoninic acid Cu^+ complex is influenced in protein samples by the presence of cysteine/cystine, tyrosine, and tryptophan side chains. The amount of protein present in a solution can be quantified by measuring the absorption spectra and comparing with protein solutions of known concentration.

2.5.3 LC-MS/MS analysis:

The bioanalytical part was carried out using the Liquid Chromatography coupled with Tandem Mass Spectrometry (LC-MS/MS).

LC-MS/MS methods were developed to quantify the following metabolites: 6-hydroxy chlorzoxazone, 4-hydroxyquinoline, 7-hydroxycoumarin glucuronide and acetylisoniazide formed during incubations with HepaRG cells and AR-CALUX® cells.

The LC-MS/MS parameters optimised for each metabolite are summarised in the following tables (**Table 3-6**).

Table 3: 6-Hydroxy chlorzoxazone					
HPLC conditions					
Column	Xterra MS C8 5 μm 4.6 x 50 mm (Waters)				
Mobile phase A	Water MilliQ+ 0.1 % formic acid				
Mobile phase B	Acetonitrile + 0.1 % formic acid				
Injection volume	10 μL				
Gradient	Step	Total time (min)	Flow ($\mu\text{L}/\text{min}$)	%A	% B
	0	0.00	400	95	5
	1	0.50	400	95	5
	2	3.50	400	5	95
	3	6.50	400	5	95
	4	6.60	400	95	5
	5	9.60	400	95	5
Retention time	6-hydroxy chlorzoxazone: 4.2 min DDIBA: .0 min				
MS conditions					
Scan type	Multiple Reaction Monitoring (MRM)				
Polarity	Negative				
Ion source	Turbo spray				
MRM Transitions	Compound	Q1-Q3	DP	CE	
	6OH-CZ	184-120	- 60	- 28	
		184-64	- 60	- 42	
		184-148	- 60	- 22	
	IS DDIBA	334.3-117	- 70	- 30	

Table 4: 4-hydroxyquinoline					
HPLC conditions					
Column	Xterra MS C8 5 µm 4.6 x 50 mm (Waters)				
Mobile phase A	Water MilliQ+ 0.1 % formic acid				
Mobile phase B	Acetonitrile + 0.1 % formic acid				
Injection volume	10 µL				
Gradient	Step	Total time (min)	Flow (µL/min)	%A	% B
	0	0.00	400	95	5
	1	0.30	400	95	5
	2	4.50	400	70	30
	3	4.60	400	5	95
	4	7.60	400	5	95
	5	7.70	400	95	5
	6	13.00	400	95	5
Retention time	4-hydroxyquinoline: 4.8 min DDIBA: 7.0 min				
MS conditions					
Scan type	Multiple Reaction Monitoring (MRM)				
Polarity	Positive				
Ion source	Turbo spray				
MRM Transitions	Compound	Q1-Q3	DP	CE	
	4-OH quinoline	146-77	110	45	
		146-104	110	32	
		146-128 *	110	30	
		146-51	110	70	
DDIBA	336-308	70	30		

Table 5: 7-Hydroxycoumarin Glucuronide					
HPLC conditions					
Column	Xterra MS C8 5 µm 4.6 x 50 mm (Waters)				
Mobile phase A	Water MilliQ+ 0.1 % formic acid				
Mobile phase B	Acetonitrile + 0.1 % formic acid				
Injection volume	10 µL				
Gradient	Step	Total time (min)	Flow (µL/min)	%A	% B
	0	0.00	400	95	5
	1	3.00	400	5	95
	2	5.00	400	5	95
	3	5.10	400	95	5
	4	8.00	400	95	5
Retention time	HCG: 3.3 minutes DDIBA: 4.5 min				
MS conditions					
Scan type	Multiple Reaction Monitoring (MRM)				
Polarity	Negative				
Ion source	Turbo spray				
MRM Transitions	Compound	Q1-Q3	DP	CE	
	HCG	337-175 *	- 80	- 15	
		337-161	- 80	- 30	
		337-113	- 80	- 20	
	IS DDIBA	334.3-317	- 70	- 30	

Table 6: Acetylisoniazide					
HPLC conditions					
Column	Atlantis Hilic Silica 3 µm 2.1 x 50 mm (Waters)				
Mobile phase A	10 mM Ammonium formate + 0.125 % formic acid				
Mobile phase B	Acetonitrile				
Injection volume	5 µL				
Gradient	Step	Total time (min)	Flow (µL/min)	%A	% B
	0	0.00	300	3	97
	1	0.30	300	3	97
	2	5.50	300	40	60
	3	7.50	300	40	60
	4	7.60	300	3	97
	5	12.00	300	3	97
Retention time	Acetylisoniazide: 4.3 min Gabapentin: 6.1 min DDIBA: 0.6 min				
MS conditions					
Scan type	Multiple Reaction Monitoring (MRM)				
Polarity	positive				
Ion source	Turbo spray				
MRM Transitions	Compound	Q1-Q3	DP	CE	
	Acetyl isoniazide	180-121 *	60	30	
		180-138	60	20	
		180-120	60	22	
		180-79	60	55	
		180-66	60	50	
	IS GABA	172-154	30	20	

3 Results

3.1 Incubation assay

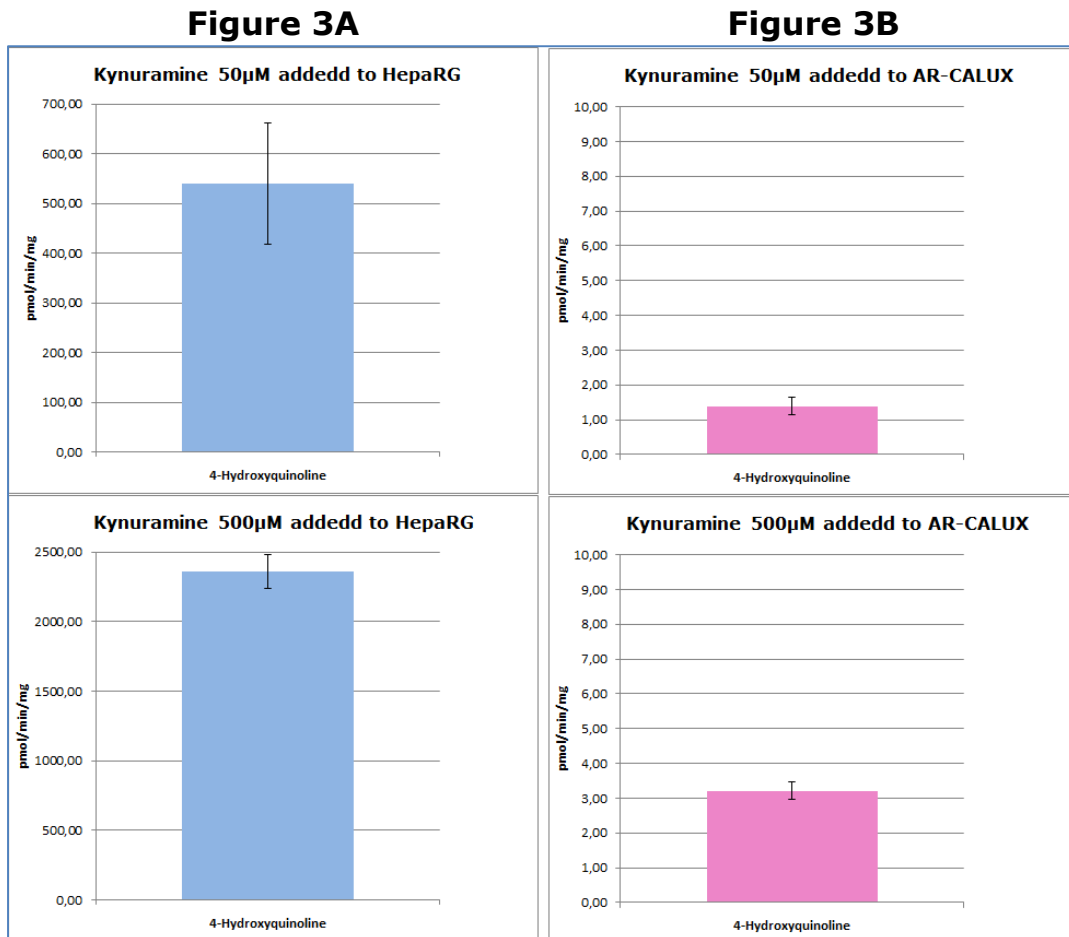
The following sections summarise the results of the presence of the four selected enzymes in HepaRG cells and AR-CALUX cells.

3.1.1 Presence of CYP2E1 enzyme:

In both cell lines, the concentration of 6-Hydroxychlorzoxazone was not detected by LC-MS/MS analysis and therefore the result is that no detectable amount of CYP2E1 is present in neither HepaRG nor AR-CULUX.

3.1.2 Presence of MAO enzyme:

Figure 3A shows the graph comparing the concentrations of 4-Hydroxyquinoline formed (which is indicative of the presence of MAO enzyme) after exposure of HepaRG to the probe substrate Kynuramine, while **Figure 3B** shows the results in AR-CALUX cells.

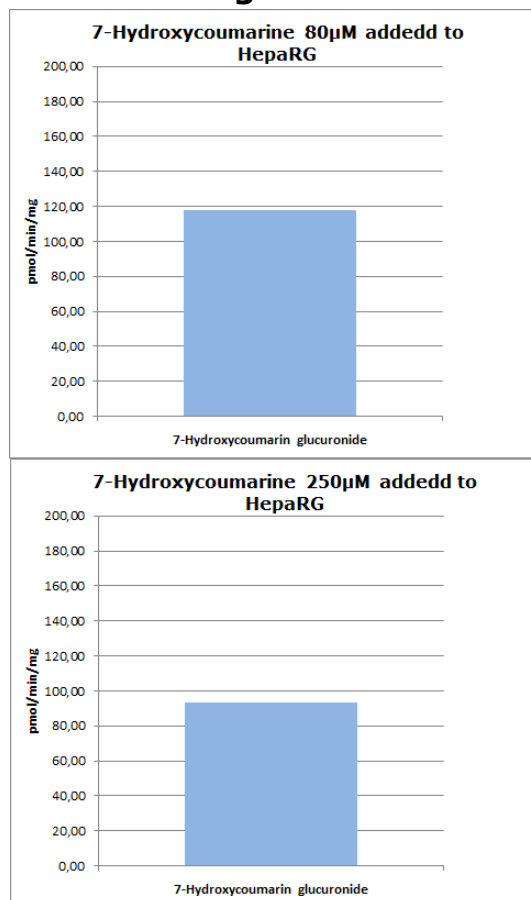


3.1.3 Presence of UGT enzyme:

Figure 4 shows the graph comparing the concentrations of 7-Hydroxycoumarin glucuronide formed (which is indicative of the presence of UGT enzyme) after exposure of HepaRG to the probe substrate 7-Hydroxycoumarin.

7-Hydroxycoumarin glucuronide was not detected by LC-MS/MS analysis in AR-CALUX and therefore the result is that no detectable amount of UGT is present AR-CULUX.

Figure 4



3.1.4 Presence of NAT enzyme:

Figure 5A shows the graph comparing the concentrations of acetyl isoniazide formed (which is indicative of the presence of NAT enzyme) after exposure of HepaRG to the probe substrate Isoniazid, while **Figure 5B** shows the results in AR-CALUX cells.



4 Conclusion

Sensitive and robust analytical methods with good linearity were developed for metabolite quantification.

The results obtained shown that cultured HepaRG cells express enzymatic activity for the following biotransformation enzymes: MAO (Phase I), UGT and NAT (both Phase II). No CYP2E1 (Phase I) could be detected. Also Kanebratt and Andersson [3] observed that the metabolism of 7-ethoxycoumarin was low, confirming low levels of CYP2E1 although not absent.

Also cultured AR-Calux cells express Phase I and Phase II activities. However, the measure activities are about 10 times lower for NAT and about 400 times lower for MAO compared to cultured HepaRG cells. No enzymatic activity is observed in AR-Calux cells for CYP2E1 (Phase I) and UGT (Phase II).

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List of abbreviations and definitions

CYP2E1: Cytochrome P450 2E1

LC-MS/MS: liquid chromatography-tandem mass spectrometry

MAO: Monoamine oxidase

NAT: N-Acetyltransferase

OECD: Organisation for Economic Co-operation and Development

UGT: Uridine 5'-diphospho-N-acetylgalactosamine glycosyltransferase

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