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Development of an *in vitro* metabolic hepatic clearance method

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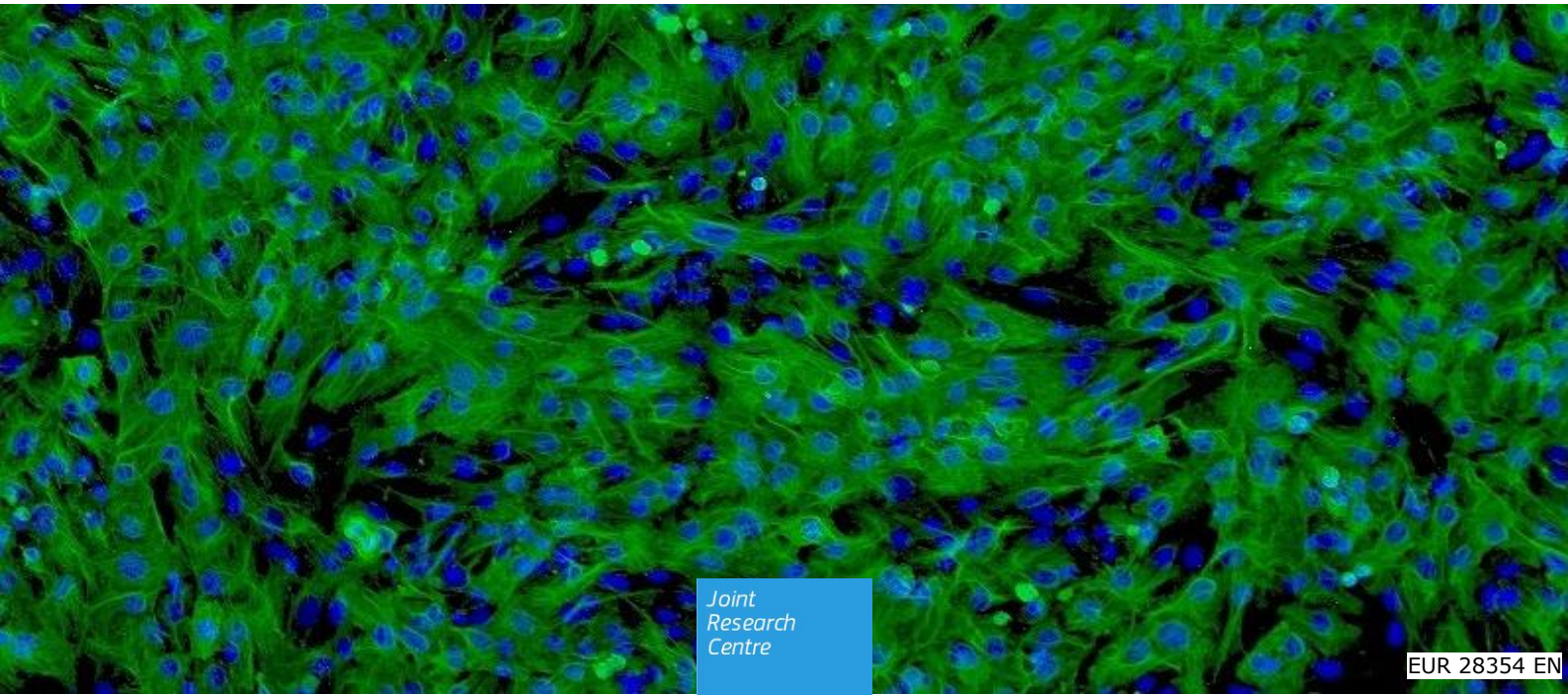
2016



Joint Research Centre

EUR 28354 EN

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JRC Science Hub
https://ec.europa.eu/jrc

JRC104213

EUR 28354 EN

PDF ISBN 978-92-79-64627-0 ISSN 1831-9424 doi: 10.2788/886451

Luxembourg: Publications Office of the European Union, 2016

How to cite: Lostia AM et al.; Development of an in vitro metabolic hepatic clearance method; EUR: 28354 EN doi: 10.2788/886451

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Abstract

There is increasing demand from *in vitro* method developers, validation bodies, *in vitro* method end-users as well as receiving authorities and OECD to integrate kinetics information in toxicity testing in order to improve chemical risk assessment. A strategic way to do this is detailed in the "EURL ECVAM strategy for achieving 3Rs impact in the assessment of toxicokinetics and systemic toxicity" (Bessems J et al. 2015). One of the strategic aims of this report urges for the development and applied use of *in vitro* kinetic methods for absorption, distribution, metabolism and excretion to be used in the future risk assessment approaches using new advanced methodologies (NAMs).

Information on metabolism has been identified as a critical piece of information in integrated test strategies based on non-animal methods. Therefore, the purpose of this report is to describe the preliminary experimental work towards a representative *in vitro* human hepatic metabolic clearance method that can be ultimately used in future defined approaches for toxicological risk assessment.

The proposed method is a result of the efforts of the EURL ECVAM laboratory team combined with knowledge gathered during (1) a literature search on *in vitro* human hepatic metabolic clearance methods, (2) an EURL ECVAM call for procedures detailing *in vitro* human hepatic metabolic clearance methods and (3) an expert meeting to establish the elements critical for *in vitro* methods that predict human hepatic metabolic clearance. The representative *in vitro* human hepatic metabolic clearance method was used to assess the *in vitro* intrinsic clearance of the following three chemicals: Acetaminophen, Diclofenac and Verapamil. Each chemical was added at one concentration to cryopreserved primary human hepatocytes (pooled from 10 donors) and the cells were exposed at different incubation time-points up to 2 h. The concentrations of each chemical over-time were measured by using Ultra-Performance Liquid-Chromatography (UPLC) coupled with (Quadrupole Time-of-Flight) Q-Tof mass spectrometer.

The results obtained show good within-laboratory reproducibility (e.g. between different runs on different days), of the calculated *in vitro* intrinsic clearance.

The results and experience gained with this experimental in-house effort by Directorate F – Health, Consumers and Reference Materials, F.3 Chemicals Safety and Alternative Methods will support the definition of an EURL ECVAM recommendation on human hepatic metabolic clearance and the subsequent drafting of a proposal for 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

Kinetics describes the concentration-time profile of a chemical in a biological system (e.g. human body, organ, tissue, cell). In other words, it describes what the body (or cell) does to a chemical and it is the opposite of dynamics which describes what a chemical does to the body (or cell).

Kinetics is the result of 4 processes: Absorption, Distribution, Metabolism and Excretion (ADME). Metabolism (chemical bio-transformation or 'metabolic clearance') is a critical factor in determining kinetic behaviour of compounds of various kinds. Metabolism can occur in several organs and tissues in the body. Nevertheless, the liver is the main site responsible for metabolic clearance.
In short, human hepatic metabolic clearance represents in many cases one of the two most important determinants of the concentration-time profile of a chemical, the other one being the human absorption kinetics. Therefore, hepatic metabolic clearance data can represent an indispensable information source to support the chemical risk assessment.

In fact, there is increasing demand (from *in vitro* method developers, validation bodies, *in vitro* method end-users as well as receiving authorities and OECD) to integrate kinetics information in toxicity testing in order to improve chemical risk assessment. (EURL-ECVAM TK Strategy, Bessems J *et al*. 2015 [**1**]).

Exposure to a chemical does not automatically mean that all of the dose will be bioavailable and therefore able to cause a specific toxicity. Hence the knowledge of the chemical concentration-time profile in a biological system (therefore the chemical kinetics) can assist to better design toxicity tests (both *in vivo* and *in vitro*) and interpret toxicological findings by:

- selecting the most relevant doses to be administered (e.g. avoid doses which can cause saturation of metabolism which then will lead to general systemic toxicity effects);
- selecting the most sensitive animal species which can provide valuable information relevant to human biology (e.g. improve the extrapolation from animal toxicological data and assess relevance for humans);
- waiving toxicity tests which can reduce the use of laboratory animals (e.g. if kinetics data indicate that a chemical bio-accumulates in the body then chronic toxicity studies are more relevant than acute-studies);
- improving the design on *in vitro* tests and the interpretation of the data generated (e.g. evaluate if *in vitro* metabolism is similar to the *in vivo* counterpart to then assess the relevance of the data when making *in vitro* to *in vivo* extrapolation).

There are several other applications where knowledge of kinetics can support and improve chemical risk assessment and a more detailed discussion can be found in EURL-ECVAM TK Strategy (Bessems J *et al*. 2015 [**1**]). In several EU chemical management legislations (REACH [**2**], CPR [**3**], PPPR [**4**], BPR [**5**]) it is required or recommended information on obtaining kinetics data. A more detailed overview was published in the EURL-ECVAM TK Strategy (Bessems J *et al*. 2015 [**1**]).

Since modern toxicology relies more on the use of alternative methods to animal toxicity testing, methods are being developed/used to provide kinetics information.

Therefore, considering that human hepatic metabolic clearance plays a key role in determining kinetics, there are already several non-guideline *in vitro* methods to measure *in vitro* human hepatic metabolic clearance and these methods can significantly vary for the experimental-settings, stage of development, intended use, reliability, relevance, etc.

Following the above considerations, the purpose of this project was to implement in EURL ECVAM laboratory facility an *in vitro* metabolic hepatic clearance method in order to build the in-house expertise to perform this type of studies which can serve multiple research works focused on supporting chemical risk assessment by using alternative to animal toxicity testing methods.

The development of an *in vitro* human hepatic metabolic clearance method (or *in vitro* clearance method) in the EURL ECVAM laboratory facility was based on previous work which is described in more details in the following two deliverables recently published:

- EURL ECVAM literature survey to identify publically available *in vitro* human hepatic metabolic clearance/ stability methods (2014) [**6**]
- EURL ECVAM web survey on *in vitro* human hepatic metabolic clearance/ stability methods (2014) [**7**]

Briefly, the EURL ECVAM established a process to map existing *in vitro* human hepatic metabolic clearance methods in order to evaluate the experimental conditions used across various settings.

The ultimate goal (which is part of the Work Programme of Directorate F – Health, Consumers and Reference Materials, F.3 Chemicals Safety and Alternative Methods and object of future planned deliverables) is to develop a Guidance Document (to be submitted to the OECD) which serves to: i) characterise and report the most important elements and attributes of *in vitro* hepatic metabolic clearance methods; ii) evaluate

their performance and iii) report all this information in a structured and easily accessible way.

The reason to have such Guidance is that there are several *in vitro* metabolic clearance methods which differ in their experimental design and for the application they are used for. Therefore there is a guidance is needed on how to evaluate if the available (or new) methods produce relevant information that can be used to support chemical risk assessment.

In order to contribute in achieving the above objective of developing a Guidance Document, the process established by EURL ECVAM to map existing *in vitro* clearance methods consisted in a collective knowledge-gathering exercise involving: a literature survey, a test submission e-survey and an expert workshop.

Based on all the information gathered covering several existing *in vitro* metabolic clearance methods, a representative *in vitro* metabolic clearance method was identified (**Figure 1**).

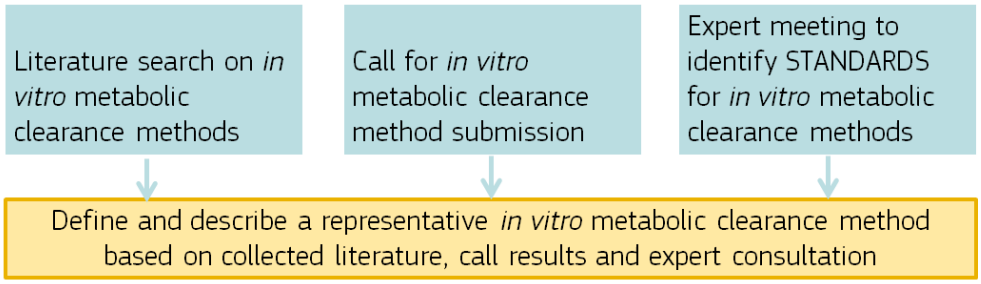


Figure 1

This representative method is not meant to be the "best possible" *in vitro* human hepatic metabolic clearance method but simply indicates the most common used experimental layout (fitted for specific purposes) built on existing experience collected from available *in vitro* clearance methods.

Therefore the representative methods can be considered as having a set of experimental elements that, based on practical experience, have proven to generate reliable and relevant data.

Before describing in the Materials and Methods section the work done to implement the representative method, the following discussion will provide a general description of possible approaches to develop *in vitro* hepatic metabolic clearance methods.

In vitro human hepatic metabolic clearance is commonly measured using hepatocytes suspension cultures (generally collected from human liver which are called "primary hepatocytes" or from cancer cells) or hepatic microsomes (sub-cellular fractions). In contrast to microsomes (representing only the Phase I biotransformation enzymes of human liver since they are lacking conjugation activities), the use of primary human hepatocytes (fresh or cryopreserved) is widely accepted as the most appropriate *in vitro* system since all the metabolic enzymes (Phase I and Phase II biotransformation enzymes) but also transporters are present in a physiological relevant set-up. A typical experiment consists in exposing the hepatocytes to one concentration of the chemical to be tested (the concentration should be below cytotoxicity) in order to determine intrinsic clearance (CL_{int}).

CL_{int} is expressed as µL/(min*millions of cells) and indicates the volume cleared by the chemical (due to metabolism) per minute and per million of hepatocytes used in the experiment. Therefore CL_{int} refers to enzyme-mediated clearance that would occur without physiological limitations (e.g. protein binding, hepatic blood flow).

In general, two approaches are used to determine CL_{int}: metabolite formation and substrate depletion. For metabolite formation, the chemical is exposed to hepatocytes at one concentration over several time-points and the formation of the metabolites is measured. This requires the knowledge of which metabolites are formed and therefore this approach is technically demanding and not always feasible when there prior knowledge of the metabolites is lacking.

The second approach, named substrate depletion, entails the incubation of the hepatocytes exposed to the test compound at one concentration and the measurement of its disappearance over time. This approach is technically easier to be performed and in fact it is the most common *in vitro* metabolic clearance method.

The method implemented in the EURL ECVAM laboratory facilities made use of a suspension culture of pooled cryopreserved primary human

hepatocytes (collected from 10 different donors). The CL_{int} was measured by using the substrate depletion approach for 3 monoconstituent substrates used as reference chemicals with available *in vivo* hepatic clearance data, e.g. acetaminophen, diclofenac and verapamil.

2 Materials and methods

2.1 General experimental layout

Plate format used for experiment	Sterile 96-well clear polypropylene plates with lid, round bottom, uncoated
***In vitro* cell model**	10 donor pool of human cryopreserved hepatocytes for suspension use
Concentrations/chemical tested *in vitro*	One concentration which is 1 µM (this is the final concentration to which cells are exposed and it is prepared in cell medium. The percentage of potential organic solvents, used to prepare chemical stock concentration, is 0.01%)
Volume of chemical to be added to cells	50 µL
Number of cells used in the incubation assay	50000 cells in 50 µL (DMEM cell medium with no serum)
Final incubation volume	100 µL (50 µL + 50 µL of cells)
Number of technical replicates	3
Incubation temperature	37° C
Incubation time points	0, 120 min and at other 4 time-points between 0 and 120 which are selected according to specific needs. (during the incubation the 96-well plate is skaked using an orbital shaker to avoid cell sedimentation
Cell viability	Check cell viability at beginning and end of incubation assay
Negative control	Cells exposed to only cell medium with no chemical
Chemicals tested	Acetaminophen, Diclofenac, Verapamil
End of incubation	At the end of each time-point, transfer 70 µL of volume to a new well and soon add 70 µL of acetonitrile containing a specific chemical internal standard. Centrifuge for 10 min at 1300 g (at 4° C) and then transfer to a glass vial for chemical analysis
Chemical analysis	Liquid chromatography coupled with mass spectrometry

2.2 Materials

2.2.1 Chemicals:

- Acetaminophen
  (CAS No. 103-90-2, Sigma-Aldrich)
- Acetaminophen-D4
  (CAS No. 64315-36-2, Sigma-Aldrich) used as internal standard for acetaminophen

- Diclofenac sodium salt
  (CAS No. 15307-79-6, Sigma-Aldrich)
- Diclofenac ^{13}C6 sodium salt 4.5 hydrate
  (CAS No. 1261393-73-0, Sigma-Aldrich) used as internal standard for diclofenac
- Verapamil hydroxychloride
  (CAS No. 152-11-4, Sigma-Aldrich)
- Verapamil-D7 Hydrochloride
  (CAS No. 1188265-55-5, LGC Standards) used as internal standard for verapamil hydroxychloride

2.2.2 Cells, cell media and additives:

- 10 donor pool of cryopreserved primary human hepatocytes for suspension use (provider Bioreclamation-ITV, X008001)
- InVitroGRO Hepatocytes Thawing medium
  (BioreclamationITV, Z99019)
- Williams' Medium E (W1878 Sigma)
- Insulin, Transferrin, Selenium (ITS) (41400045 Gibco)
- L-Glutamine 200mM (25030149 Gibco)
- Hepes, (1M, pH 7.4) (Gibco 15630080)

2.2.3 Plastic ware and disposable:

- Sterile 96-well clear polypropylene plates with lid, round bottom, uncoated
- Sterile 15-mL polypropylene centrifuge tubes
- Sterile 50-mL polypropylene centrifuge tubes
- Sterile polypropylene reagent reservoir of at least 20 mL size
- Adjustable pipette 2-20 µL
- Adjustable pipette 10-100 µL
- Adjustable pipette 100-1000 µL
- Sterile pipette tips

2.2.4 Technical equipment:

- Water bath able to maintain a temperature of 37°C
- Cell incubator with 5 ± 1% CO_2 atmosphere, 37± 2°C temperature and with water-saturated atmosphere
- Orbital shaker able to operate at water-saturated atmosphere
- Balance with at least 0.01 mg readability
- Centrifuge appropriate for 96-well plates and able to maintain a temperature of 4 ^{o}C
- Heating plate with internal regulator to maintain 37^{o}C

2.2.5 Reagents used for chemical analysis:

- Acetonitrile (UPLC grade)
- Formic acid (UPLC grade)
- Ultrapure water 18.2MΩ.cm

2.2.6 Analytical equipment

UPLC Acquity coupled with Xevo G2-S QTof (Waters)

2.3 Preparation of cell assay medium, stock and working solutions

The following sections describe the preparation of the different reagents and solutions used to perform the *in vitro* metabolic hepatic clearance method.

Table 1 summarises the reagents and solutions to be prepared and the time for their preparation.

Table 1

To be prepared the day before the experiment
The cell medium to be used for incubation assay (section 2.3.1)
The mixture of cell medium and acetonitrile (50:50) (section 2.3.2)
The stop solutions with specific internal standard at 1 µM (section 2.3.3)
The5 mL stock concentration of 10 mM for each chemical to be tested (section 2.3.4.)
To be prepared the day of the experiment
The working concentration of 2 µM for each chemical to be tested (section 2.3.5)
The chemical standards for the calibration curve for LC-MS analysis (section 2.3.6)

2.3.1 Cell medium for incubation assay:

In a bottle containing 500mL of Williams's Medium E the following steps were done:

- Addition of 0.5mL of ITS (Insulin, Transferrin, Selenium)
- Addition of 5mL of L-Glutamine 200mM
- Addition of 7.5mL of HEPES
- Thorough shaking and labelling as "Cell medium for incubation assay"
- An expiry date of 1 month was assigned

2.3.2 Cell medium and acetonitrile mixture (50:50):

To obtain the mixture the following procedure was adopted:

- Addition of 10mL of "Cell medium for incubation assay" into a 20mL volumetric flask and making up to the mark with acetonitrile
- Thorough shaking and labelling as “Cell medium-acetonitrile 50:50”
- An expiry date of 1 day was assigned

2.3.3 Stop solutions using specific internal standards

Note: For each of the 3 chemicals tested, a specific internal standard was used to then performed the chemical analysis by using mass spectrometry

- To have a 2mM concentration, a defined amount of mg of the desired Internal Standard was weighted and transferred in a 5mL volumetric flask
- About 3mL of acetonitrile (or another solvent depending on the solubility) were added and checked to ensure that all the powder was dissolved. Then making up to the mark with acetonitrile
- Then a thorough shaking was done and after transferred in a glass tube and labelled as "Internal standard (either for Acetaminophen, Diclofenac or Verapamil) 2mM in acetonitrile"
- An expiry date of 6 months was assigned
- In a 10mL volumetric flask was added acetonitrile (about 9mL) until almost the mark
- 5µL of "Internal standard 2mM in acetonitrile" were added in the volumetric flask, shaken thoroughly and made up to the mark with acetonitrile
- Finally, an "Internal standard 1µM in acetonitrile" label was applied and an expiry date of 6 months was assigned

2.3.4 Stock concentration of 10mM for each chemical to be tested

- In order to have a stock solution of 10mM concentration, a defined amount of mg of the desired chemical was weighted and transferred in a 5mL volumetric flask
- About 3mL of acetonitrile were added and checked to ensure that all the powder was dissolved. Then maked up to the mark with acetonitrile
- Thoroughly shaken and transferred in a glass tube and labelled as "Chemical Name (either Acetaminophen, Diclofenac or Verapamil) 10mM in acetonitrile"
- An expiry date of 6 months was assigned

2.3.5 Working concentration of 2µM for each chemical to be tested

Note: the working concentration was the one to which the cells were exposed for the incubation assay to measure *in vitro* metabolic hepatic clearance.
When the working concentration was added to the cells, the final concentration was 1µM. It was also important that the percentage of organic solvent was less than 0.1% to avoid potential interference with the cells. In this case the percentage of acetonitrile was 0.01% when added to the cells.

- Using a calibrated glass syringe, 200µL of a specific chemical stock concentration of 10mM were transferred in 1mL volumetric flask, and then made up to the mark with "cell medium-acetonitrile 50:50" solution.
- After a thorough shaking, the solution was transferred in a glass tube and labelled as "Chemical Name (Acetaminophen, Diclofenac or Verapamil) 2mM". An expiry date of 1 day was assigned.
- Using a calibrated glass syringe, 5µL of "Chemical Name 2mM" previously prepared were transferred in 5mL volumetric flask, and then made up to the mark with "cell medium for incubation assay" solution.
- Shaken thoroughly, the solution was then transferred in a glass tube and labelled as "Chemical Name 2µM in cell medium". An expiry date of 1 day was also assigned.

2.3.6 Analytical standards for the calibration curve for the LC-MS analysis

From each chemical working concentration at 2µM (Acetaminophen, Diclofenac or Verapamil), see section 2.3.5, serial dilutions in Williams' Medium E were made to obtain the following concentrations: 1000, 500, 250, 100, 50 and 10 nM. These concentrations were prepared in glass vials and then used to build the calibration curve. **Table 2** shows the procedure used to prepare the standards.

Table 2

Concentration to be prepared (nM)	Volume to be prepared (µL)		Volume used of more concentrated solution (µL)		Volume of cell medium added (µL)
1000	1000	=	500 (of 2µM)	+	500
500	1000		500 (of 1000 nM)		500
250	1000		500 (of 500 nM)		500
100	1000		100 (of 1000 nM)		900
50	1000		100 (of 500 nM)		900
10	1000		100 (of 100 nM)		900

2.4 Human *in vitro* hepatic metabolic clearance method

2.4.1 Study design of the *in vitro* human hepatic metabolic clearance method (or *in vitro* incubation assay)

The incubation assay consisted of exposing cryopreserved pooled primary human hepatocytes to 1 µM of Acetaminophen, Diclofenac or Verapamil at the following time-points: 0, 15, 30, 60, 90 and 120 min.

At the end of each time point, the incubation reaction is stopped by adding a solution of acetonitrile containing a specific Internal Standard (either for Acetaminophen, Diclofenac or Verapamil). After centrifugation, a certain volume of supernatant is collected, transferred to a glass vial for the following chemical analysis by mass spectrometry.

The whole incubation assay was done manually by using 96-well clear polypropylene plates.

In one experiment, all the three chemicals can be tested in the same plate following the layout described below. **Figure 2** shows the schematic representation of how the samples are organised in a 96-well plate.

The plate layout shown in **Figure 2** was then used for each of the time-points (0, 15, 30, 60, 90 and 120 min). In order to check that cell viability was not influenced by addition of the chemical, cell count was normally measured at the beginning and end of incubation assay (in this case 0 and 120 min). For this experiment, cell count was also measured at the other time-points (15, 30, 60 and 90 min) in order to monitor the number of cells during the whole incubation period.

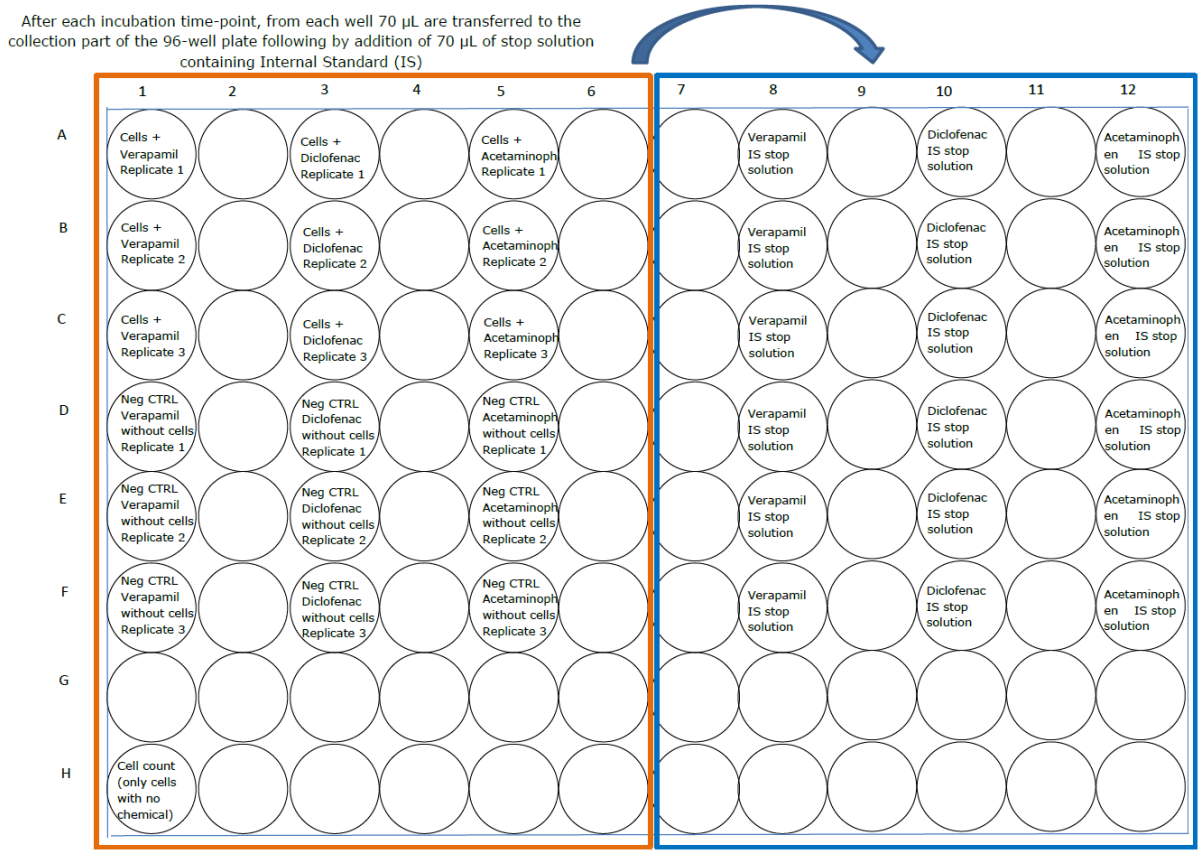


Figure 2

2.4.1.1 Prepare equipment for incubation assay

- 6 sterile 96-well clear polypropylene plates with lid, round bottom, uncoated were taken. For each plate, the lid was labelled writing the corresponding incubation time (e.g. 0, 15, 30, 60, 90 or 120 min)
- To each plate, 50µL of "Verapamil (or another chemical) working concentration of 2µM" (see section 2.3.5) were added to 3 wells, as shown in Figure 2. The position of these wells, which were used as technical replicates, was marked
- To each plate, 50µL of "Verapamil (or another chemical) working concentration of 2µM" (see section 2.3.5) and then 50µL of cell medium (see section 2.3.1) were added to 3 wells, as shown in Figure 2. Also in this case, the position of these wells which were used as negative controls was marked
- All 96-well plates were put in the incubator at 37°C and 5% CO2 for 10-15min prior to cell addition

2.4.1.2 Thawing and reconstitution of cryopreserved human hepatocytes

- The cell medium was pre-warmed (see section 2.3.1) to 37°C for about 20-30min

The following steps have taken about 30 min.

Notes:

* The thawing procedure is stressful to frozen cells, and working quickly ensures that a high proportion of the cells survive the procedure.
* 1 vial contained about 5 million of frozen hepatocytes. Since, for the incubation assay, each well contained 50000 cells, plus 3 chemicals in triplicate, 6 time points and controls (50000 x 3 chemicals x 3 replicates x 6 time points = >2700000 cells), 1 vial was enough to perform an experiment following the layout shown in Figure 2.

- 48mL of thawing medium (see section 2.2.2) were transferred to a sterile 50mL polystyrene tube
- The thawing medium was prewarmed to 37°C for about 10min
- After, the vial from the shipping container or cryostorage was carefully removed. If the vial has been stored in the liquid phase, it is necessary to ensure that liquid nitrogen is removed from the vial before warming up and check that the cap is firmly closed.
- The vial was immersed into a 37°C water bath and gently shaken for about 1.5 minutes until the ice was detached from the plastic

- The vial was transferred into the laminar flow hood and disinfected with an absorbent paper containing isopropanol or ethanol
- From the vial, all hepatocytes cell suspension were transferred into the pre-warmed 50mL polystyrene tube containing 48mL of thawing medium
- From the 50mL polystyrene tube, 1mL of solution was used to rinse the vial once to take what remains of cells. Then, transferred back into the 50mL polystyrene tube
- The hepatocytes suspension was reached by gently inverting the 50mL polystyrene tube 3 times
- The 50mL polystyrene tube was centrifuged at 50g at room temperature during 5 minutes
- The supernatant was discarded by either pouring in one motion (partial pouring and re-inverting the centrifuge tube is inadequate), or aspirating using a vacuum pump
- 4mL of pre-warmed cell medium were added to the 50mL polystyrene tube. Then, a pipette 100-1000µL was used to pipette gently for loosening the pellet
- The total cell count and the percentage of viable cells were determined using the Trypan Blue exclusion method
- After determination of the cell concentration (expressed as cells/mL), a dilution with pre-warmed cell medium was made to obtain the desired concentration of cells (for the incubation assay 50000 cells in 50µL are needed. Therefore the desired final concentration is 1 million cells in 1mL of cell medium)

Acceptance criteria to use hepatocytes for incubation assay

Two conditions must be taken into account when performing the incubation assay:

- A minimum cell viability of 80% after thawing must be obtained.
- After reconstitution, cell suspensions can be used for up to 4 hours.

2.4.1.3 Incubation assay

- Firstly, from the incubator, only the 96-well plate labelled on the lid as "0min" was removed
- 50µL of viable hepatocytes (1 million cells/mL) were added only to the 3 wells used as technical replicates (see section 2.4.1.1)
- Immediately, from each of the 6 wells of the first column containing Verapamil-exposed samples (see Figure 2), 70µL were transferred, by using a multichannel pipette, into the corresponding new column as shown in Figure 2. Then, 70µL of

"stop solution with Verapamil Internal Standard (see section 2.3.3) were immediately added to each well

- The previous step was repeated for the remaining two chemicals: Diclofenac and Acetaminophen and consequently followed the complete procedure as described below
- The 96-well plate was centrifuged at 1300g for 10min at 4°C. Then, from each well 100µL of supernatant were transferred in the corresponding previously-labelled glass vials which were stored at low temperature (-70°C) until LC-MS analysis
- From the incubator, all the remaining 96-well plates were removed and placed on the heating plate (those labelled on the lid as "15, 30, 60, 90 and 120 min")
- In order to start the incubation reaction, 50µL of viable hepatocytes (1 million cells/mL) were added, only to the wells used as technical replicates (see Figure 2). Furthermore, 50µL of viable hepatocytes were added to each plate, into one well which was used for cell-count (Figure 2)
- The 96-well plates were returned to the orbital shaker in the incubator at 37°C and the shaker speed was adjusted at 300 rpm
- At each time-point of 15, 30, 60, 90 and 120 min, the corresponding 96-well plate was removed from the incubator.
- Immediately, from each of the 6 wells of the first column containing Verapamil-exposed samples (see Figure 2), 70µL were transferred, by using a multichannel pipette, into the corresponding new column as shown in Figure 2. Then, 70µL of "stop solution with Verapamil Internal Standard (see section 2.3.3) were immediately added to each well
- The previous step was repeated for the remaining two chemicals: Diclofenac and Acetaminophen

Note: it was important to record the exact time when the stop solution was added to the samples

- The 96-well plate was centrifuged at 1300g for 10min at 4°C. Then, 100µL of supernatant from each well were transferred in the corresponding previously-labelled glass vials which were stored at low temperature (-70°C) until LC-MS analysis.

2.4.1.4 Preparation of analytical standards

For each chemical, the prepared standards (see section 2.3.6) were obtained following the same procedure used for the preparation of the samples as described in section 2.4.1.3.

Briefly, from each standard 70µL were transferred to a new glass vial, previously labelled. Then 70µL of "stop solution with corresponding Internal Standard (see section 2.3.3)" were added. The glass vials were

then centrifuged at 1300g for 10min at 4°C. Then, 100μL of supernatant were transferred in a new previously-labelled glass vials which were stored at -70°C until LC-MS analysis.

2.4.2 LC-MS/MS analysis

The mass spectrometer was a Xevo G2-S QTof (Waters) coupled with an Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters) and interfaced with an ElectroSpray Ion source (ESI).
For all 3 chemicals the column used was a 50×2.1mm, Acquity UPLC-BEH C18 1.7μm. Mobile phase A was an aqueous solution of 0.1% formic acid and as mobile phase B was used 100% acetonitrile containing 0.1% of formic acid.
The chromatographic conditions for Diclofenac and Verapamil were the same as shown in **Table 3**.

Table 3

Time (min)	Flow rate (mL/min)	A (%)	B (%)
0	0.4	98	2
0.5	0.4	98	2
2.5	0.4	20	80
2.51	0.4	2	98
4.5	0.4	2	98
4.51	0.4	98	2
6	0.4	98	2

The chromatographic conditions for acetaminophen are shown in **Table 4**.

Table 4

Time (min)	Flow rate (mL/min)	A (%)	B (%)
0	0.4	98	2
0.4	0.4	98	2
2.2	0.4	2	98
3	0.4	2	98
3.1	0.4	98	2
4	0.4	98	2

For Diclofenac and Verapamil the instrument operated in MS/MS mode in which the parent ion was selected in the quadrupole and then fragmented. The resulting product ions were monitored for the quantitative analysis. For Acetaminophen the instrument operated in MS^e mode and no specific product ions were selected for the quantitative analysis.

Table 5 summarises the source parameters optimised for each chemicals.

Table 5

Instrumental Parameters Settings	Test Chemical		
	Acetaminophen	**Diclofenac**	**Verapamil**
Polarity ionisation	Positive	Negative	Positive
Capillary (kV)	1	0.5	0.4
Cone (V)	40	100	40
Source Temperature (°C)	120	120	120
Desolvation Temperature (°C)	350	500	500
Cone Gas Flow (L/h)	50	50	50
Desolvation Gas Flow (L/h)	1000	1000	1000

Table 6 summarises the precursor- and product-ion(s) monitored for each chemical and used for the quantitative analysis. Diclofenac quantification, the precursor/product ion pair m/z 250/214 was used as quantifier, while the pair at m/z 455/303 was used for verapamil.

Table 6

Chemical	Polarity	MS mode	Precursor ion (m/z)	Product ion (m/z)
Acetaminophen	positive	MS[e]	151.06	-
Acetaminophen-D4	positive	MS[e]	155.08	-
Diclofenac	negative	MS/MS	250.01	178.06
				214.04
Diclofenac ^{13}C6	negative	MS/MS	256.08	220.11
Verapamil	positive	MS/MS	455.29	165.09
				303.20
Verapamil-D7	positive	MS/MS	462.33	310.29

2.4.3 Calculation of *in vitro* intrinsic clearance (CL_{int})

The calculated concentrations, expressed as nM, have been transformed into a natural logarithmic scale (ln).
Then the elimination rate constant (k) was calculated from the slope of $\ln(C_t/C_0)$ vs t (min), with a 1/y weighting, according to equation 1.

$$\ln(\frac{Ct}{C0}) = -kt \qquad \text{(eq. 1)}$$

C_t = the substrate concentration in the incubation well at time t (min)

C_0 = the substrate concentration in the incubation well at time t= 0

k = the elimination rate constant

The intrinsic clearance *in vitro* (CL_{int} *in vitro*) was calculated according to the following equation:

$$CLint = \frac{kV}{N} \qquad \mu L/ (min*millions\ of\ cells)$$

Where:

$k = \frac{0.639}{t1/2}$

V = incubation volume (100 µL)

N = number of cells ($x10^6$) in the incubation (50000 cells used in this assay)

3 Results

3.1 LC-MS analysis

Verapamil and diclofenac were eluted at 2.10 and 2.65 min, respectively while the retention time of acetaminophen was eluted at 1.21 min.
Figure 3 shows the chromatogram for each chemical.

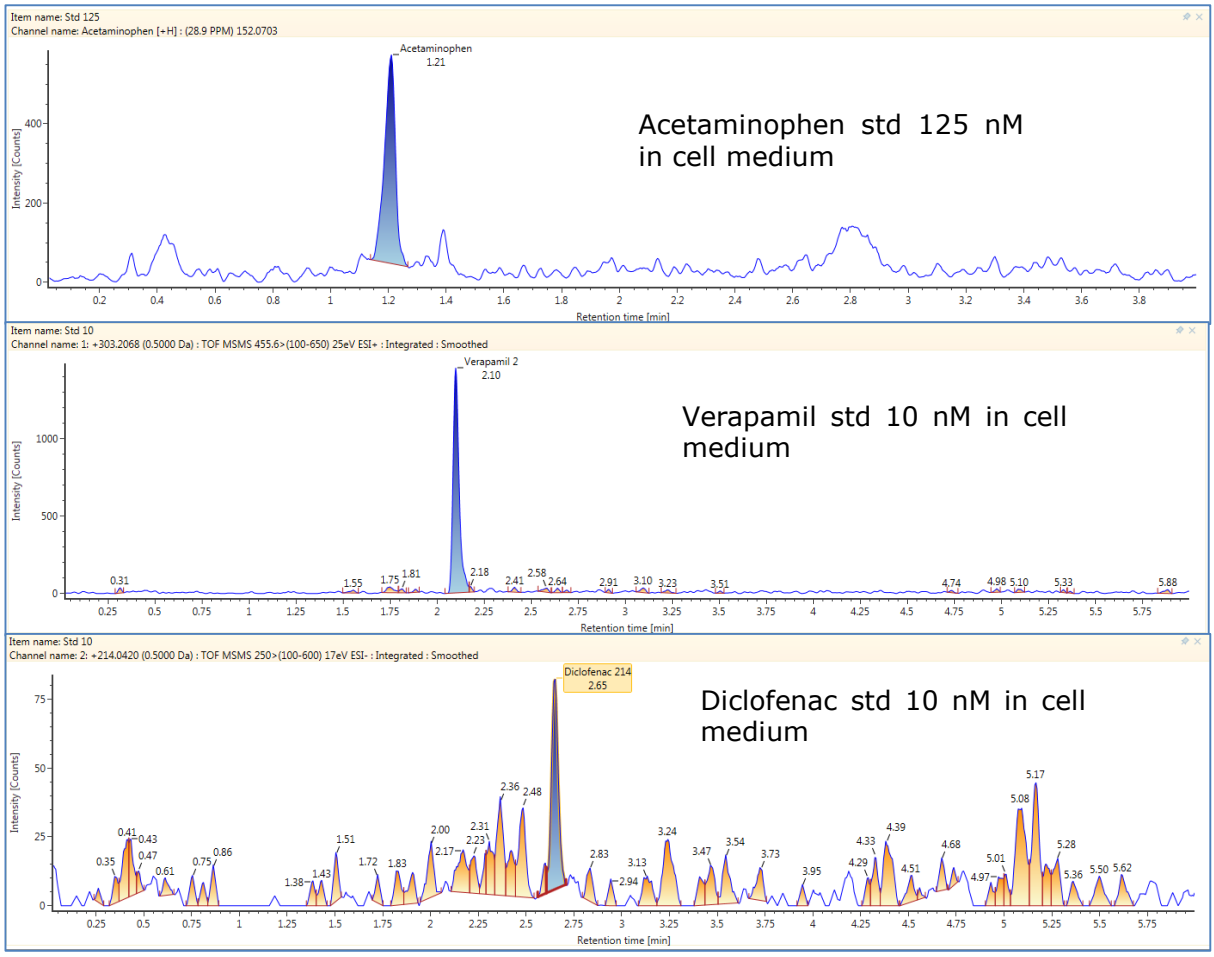


Figure 3

A linear response was observed in the range 10–1000 nM in cell medium for all 3 chemicals. For all chemicals, the limit of quantification (LOQ) is 10 nM while the limited of detection (LOD) is 1 nM.
Between- and within-day precision and accuracy were always below 20% for both the analytes and no carry-over was observed (data not shown).

3.2 *In vitro* hepatic metabolic clearance

Following the experimental protocol described in section 2.4.1, three experiments were performed by exposing cells to Diclofencac or Verapamil and two experiments by adding Acetaminophen to the cells.

In each experiment, samples were run in triplicate (as shown in **Figure 2**).

The reproducibility between experiments was good as shown in **Figure 4, 5 and 6 and Table 7**.

The concentration of Diclofenac and Verapamil decreased over time indicating that these two chemicals were bio-transformed.

Regarding acetaminophen, no significant difference in concentration over time was observed within the 2 h exposure.

Figure 4

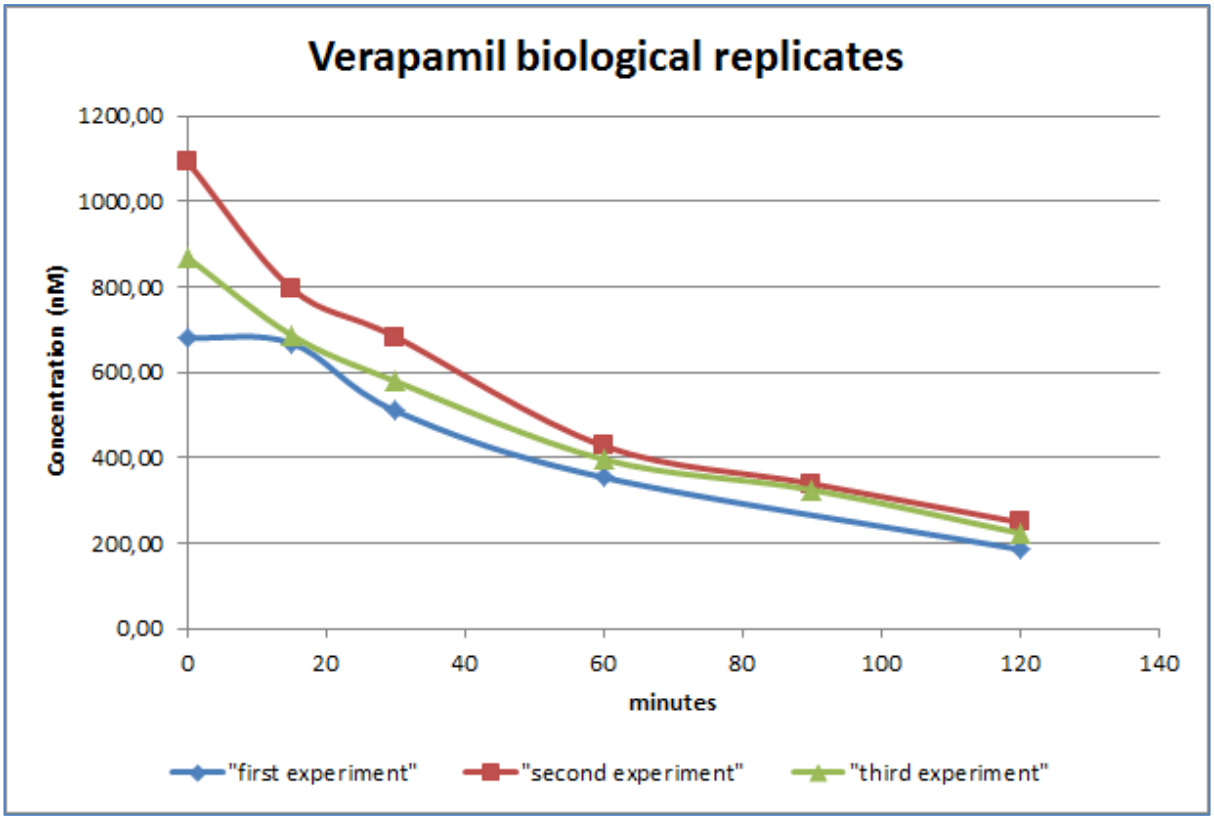


Figure 5

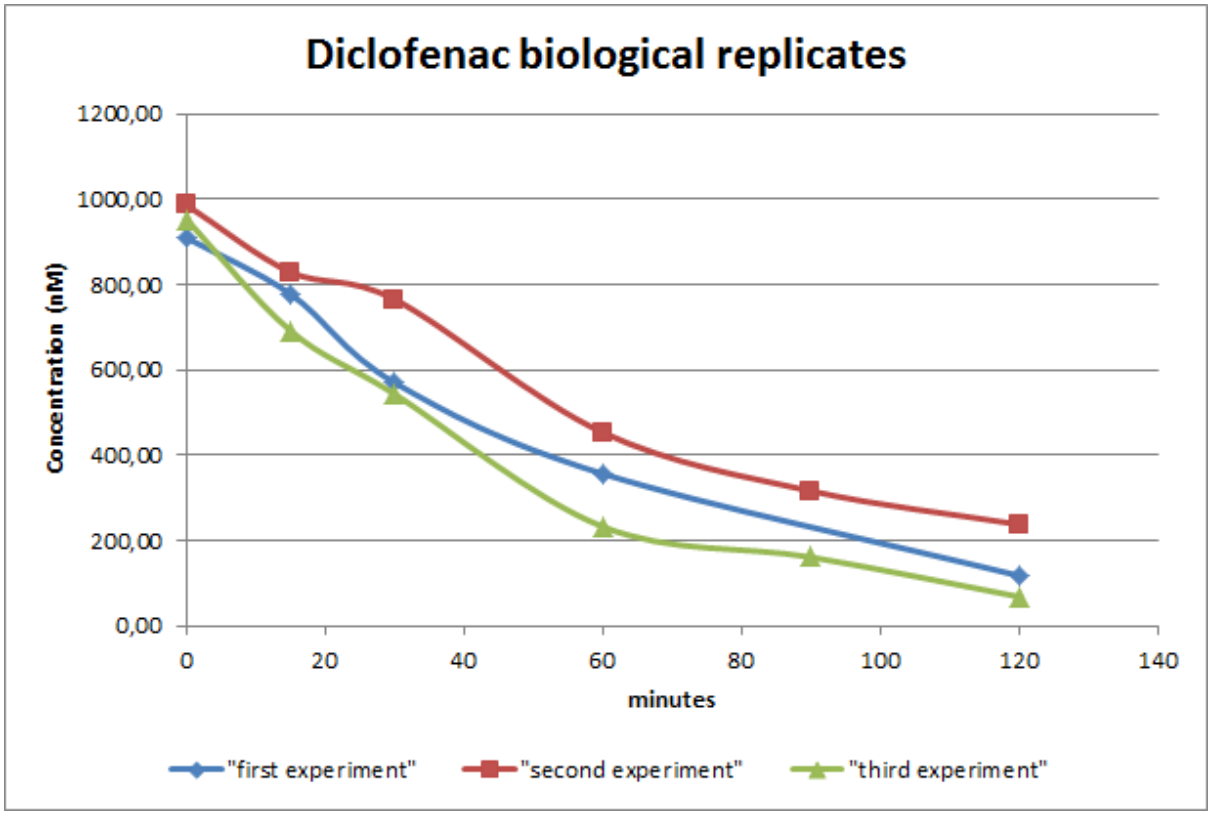
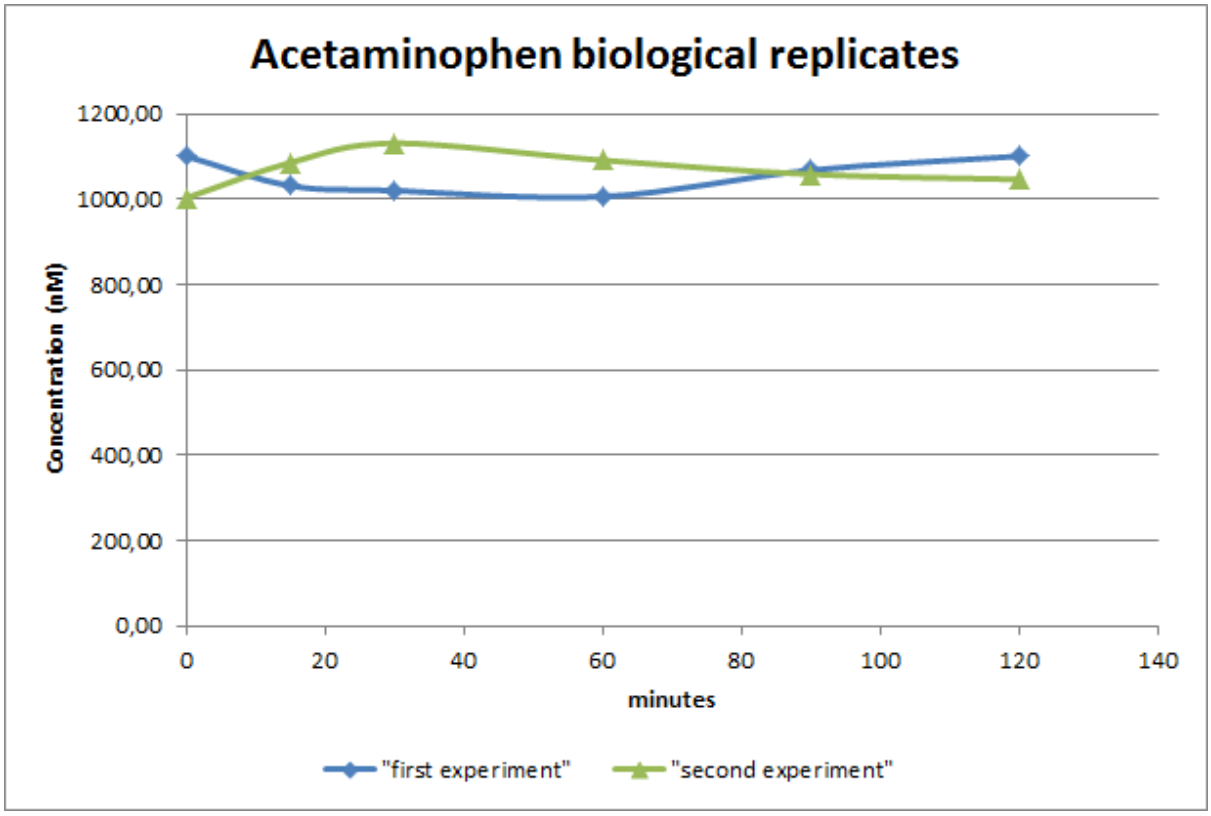

Diclofenac biological replicates
1200,00
1000,00
800,00
600,00
400,00
200,00
0,00
Concentration (nM)
0
20
40
60
80
100
120
140
minutes
"first experiment"
"second experiment"
"third experiment"

Figure 6


Acetaminophen biological replicates
1200,00
1000,00
800,00
600,00
400,00
200,00
0,00
Concentration (nM)
0
20
40
60
80
100
120
140
minutes
"first experiment"
"second experiment"

The values of intrinsic clearance (calculated following equations in section 2.4.3) for diclofenac and verapamil are shown in **Table 7**.

Verapamil biological replicates						
	First experiment		Second experiment		Third experiment	
Time (min	Average concentration (uM) across 3 technical replicates	SD	Average concentration (uM) across 3 technical replicates	SD	Average concentration (uM) across 3 technical replicates	SD
0	681,08	58,99	1093,00	20,68	869,42	31,72
15	668,22	12,88	796,78	16,66	687,20	5,42
30	509,75	2,15	683,12	21,21	579,05	16,54
60	354,09	5,68	427,65	12,65	396,34	8,29
90			339,46	8,54	325,07	3,80
120	185,66	11,88	248,79	20,84	223,76	
Intrinsic clearance value, µL/ (min*millions of cells)	22,8		24,00		21,80	

Diclofenac biological replicates						
	First experiment		Second experiment		Third experiment	
Time (min	Average concentration (uM) across 3 technical replicates	SD	Average concentration (uM) across 3 technical replicates	SD	Average concentration (uM) across 3 technical replicates	SD
0	909,67	36,23	986,51	11,57	952,71	94,28
15	777,67	14,05	829,29	52,08	692,13	88,30
30	570,33	13,32	765,27	25,03	543,24	29,38
60	356,67	32,35	453,41	15,64	232,76	7,13
90			316,36	11,82	161,46	26,11
120	118,00	8,19	238,12	9,77	68,66	14,94
Intrinsic clearance value, µL/ (min*millions of cells)	34,6		24,8		43,00	

Acetaminophen biological replicates						
	First experiment		Second experiment			
Time (min	Average concentration (uM) across 3 technical replicates	SD	Average concentration (uM) across 3 technical replicates	SD		
0	1100,62	50,96	1003,19	43,54		
15	1031,46	74,23	1085,77	49,13		
30	1020,02	8,59	1131,01	23,52		
60	1005,98	30,85	1091,17	83,36		
90	1068,12	32,25	1058,51	4,61		
120	1100,62	51,16	1046,46	37,84		
Intrinsic clearance value, µL/ (min*millions of cells)	0,4		0,04			

4 Conclusion

Knowledge of *in vitro* human hepatic metabolic clearance can improve the prediction of chemical's toxicity by using alternatives to animal testing methods and therefore better support chemical risk assessment.

This report describes the preliminary work done at the EURL ECVAM laboratory facility to describe an experimental procedure towards a representative *in vitro* human hepatic metabolic clearance method that can be ultimately used in future defined approaches for toxicological risk assessment.

The method implemented was based on a collective knowledge-gathering exercise in which existing available *in vitro* human hepatic metabolic clearance methods were mapped and compared. The method implemented, represented the most common experimental features used by the existing methods gathered while doing the mapping exercise.

As preliminary work, three chemicals with known *in vivo* human hepatic metabolic clearance data were used to challenge the *in vitro* method implemented in the EURL ECVAM laboratory.

The scope of the project was to evaluate the reproducibility of the data (concentration-time curves and calculated *in vitro* intrinsic clearance) between the different experimental runs performed.

The data obtained clearly show good reproducibility.

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List of abbreviations and definitions

ADME: Absorption, Distribution, Metabolism, Excretion
BPR: Biocidal Products Regulation
CL_{int}: intrinsic clearance
CPR: Cosmetic Products Regulation
LC-MS: liquid chromatography-mass spectrometry
NAMs: new advanced methodologies
OECD: Organisation for Economic Co-operation and Development
PPPR: Plant Protection Products Regulation
Q-Tof: quandrupole-time of flight
REACH: Registration, Evaluation, Authorisation and Restriction of Chemicals
TK: toxicokinetics
UPLC: ultraperformance liquid chromatography

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LB-NA-28354-EN-N

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doi: 10.2788/886451
ISBN 978-92-79-64627-0