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# Determination of *in vitro* metabolic hepatic clearance of valproic acid (VPA) and five analogues by UPLC-MS-QTOF, applicable in alternatives to animal testing☆

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ABSTRACT

Laboratory measurements of intrinsic clearance support the development of TK models, with potential relevance to weight of evidence toxicity assessments of xenobiotics, including read-across, the concept of predictive estimation by data extrapolation between chemicals of similar structure (analogues).

In this work a procedure with analytical method for determination of *in vitro* hepatic metabolic clearance, relevant to biotransformation toxicokinetic (TK) modelling, is presented. Cryopreserved primary human hepatocytes represent a suitable cells, due to their biological characteristics, for providing an *in vitro* model for simulating *in vivo* metabolic clearance.

The experimental part considered an adequate sequential time-frame for collecting samples and controls for all chemicals tested, including centrifugation and aliquoting of the corresponding fractions until the instrumental session.

For the first time, *in vitro* hepatocyte intrinsic clearance was measured for six analogue test chemicals: valproic acid, 2-ethyl caproic acid, octanoic acid, valeric acid, 2-methyl butyric acid and 2-trans pentenoic acid, during incubated cell culture exposure up to 2 h or 3.5 h. The time dependence of any metabolism was determined from analysis of the supernatant at intervals using a new developed analytical method for UPLC coupled with QTOF mass spectrometer. The chemicals could then be ranked by their relative intrinsic clearance.

The analyses were reproducible, with coherence of the calculated *in vitro* intrinsic clearance between experiments.

## 1. Introduction

The toxicological effects and fate of xenobiotic substances, such as manufactured products, pharmaceuticals and pesticides, are characterised mechanistically by toxico-kinetics (TK) defined physiologically by absorption, distribution, metabolism and excretion (ADME). In particular, enzymatic metabolism essentially determines biotransformation of extraneous chemicals, principally mediated in the liver, also responsible for their elimination by hepatic metabolic clearance.

In the context of regulatory safety assessment of chemicals (e.g., [3]; etc.) and obligation to alleviate dependence on animal studies [8,2]. TK modelling of ADME via *in vitro* measurement and/or *in silico* simulation

*Abbreviations:* ADME, Absorption Distribution Metabolism Excretion; BEH, Bridged Ethylene Hybrid; $CL_{int}$, Intrinsic clearance; ESI, Electrospray ionisation; ET-CAPRO, 2-ethylhexanoic or 2-ethylcaproic acid; EURL ECVAM, European Union Reference Laboratory for Alternatives to Animal Testing; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IS, Internal Standard; ITS, Insulin-Transferrin-Selenium; LC, Liquid Chromatography; LOD, Limit of Determination; LOQ, Limit of Quantification; M.W., Molecular Weight; *m/z*, mass to charge relationship; MBA, 2-methylbutyric acid; MRM, Multiple Reaction Monitoring; MS, Mass Spectrometry; NC, Negative Control; OCTA, octanoic or caprylic acid; QC, Quality Control; Q-TOF, Mass spectrometer that couples a quadrupole to a time-of flight mass analyser; R, Replicate; RSD, Relative Standard Deviation; SD, Standard Deviation; TI, Test Item; TK, Toxicokinetics; TPA, *trans*-2-pentenoic acid; UPLC, Ultra-High Performance Liquid Chromatography; VLA, valeric or pentanoic acid; VPA, valproic acid.

☆ *The authors dedicate this paper in memoriam to Dr. Alfonso Maria Lostia.*

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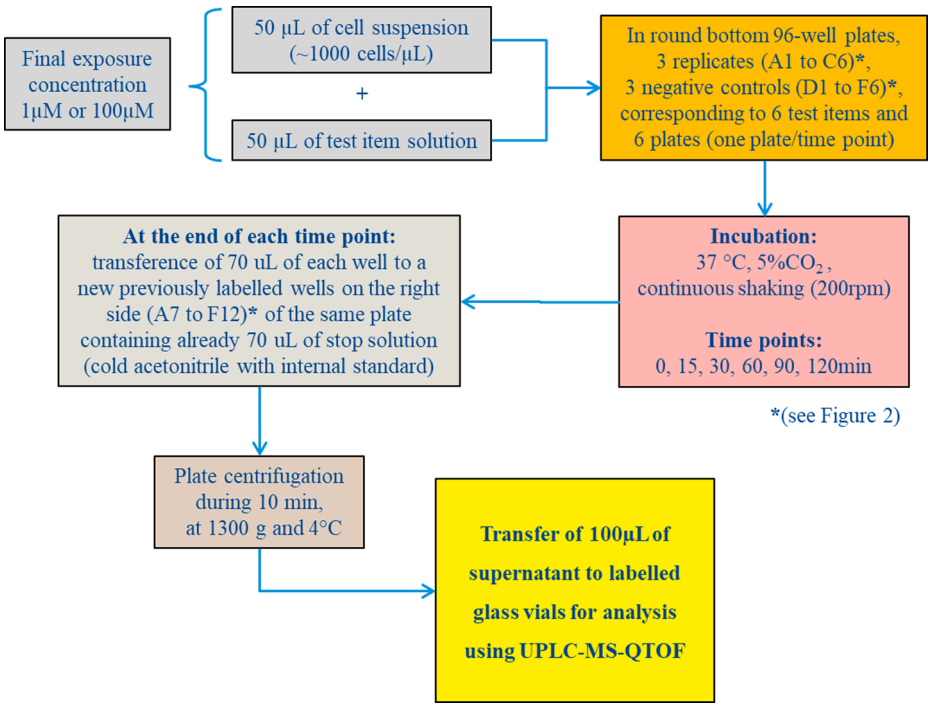
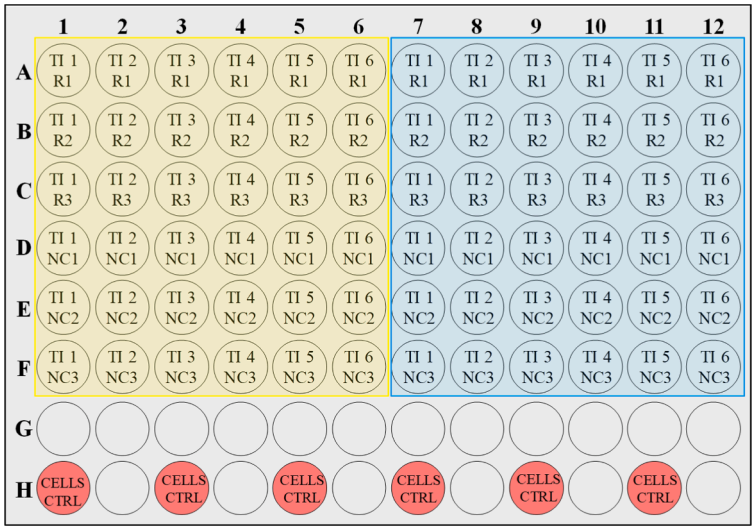



**Fig. 1.** Experimental design.

allows systematic quantitative *in vivo* prediction. Moreover, with reliable evaluation, opportunity occurs for read-across prediction by analogue extrapolation.

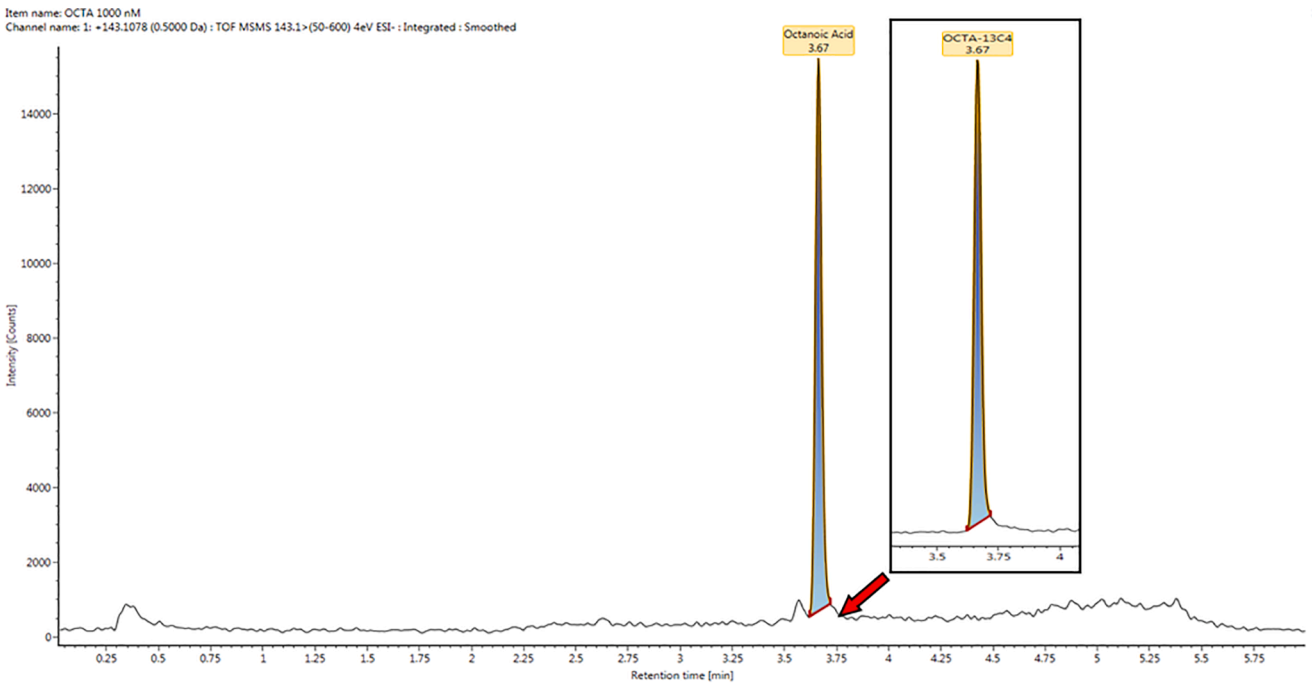
The potential application of read-across in chemical safety assessment was an objective of the international SEURAT-1 programme [11]. This approach considered four adverse effect mode of action scenarios, with aim to obtain practical evaluation, based on case studies of relevant selected chemicals [4]. Essentially, the suggested scenarios for analogue grouping were chemicals having: 1) toxicity without metabolism, 2) toxicity due to similar metabolites, 3) low or negative toxicity, and 4) variable toxicity but similar molecular structures. And for valid read-across within a selected analogue group, at least one reference or source chemical would be required, eligible by documented mode of action.

This paper reports experimental hepatocyte metabolic clearance results from laboratory *in vitro* assay. In this case, one source substance, the valproic acid (VPA), is used to be compared with five structural analogue compounds (target substances). The source and the target substances considered were short-chain carboxylic fatty acids, identified by Berggren et al. [4] and covered the four scenarios with emphasis on specificity rather than potency. Then, the "one-to-many read-across" technique can be applied to fill some data gaps [6,7].




R = replicates; NC = Negative controls
Yellow area = Test items in assay medium with (R) or without (NC) cells.
Blue area = stopped biological activity from the yellow area by addition of internal std. in ACN.
CELLS CTRL (in red) = Cells in assay medium alone for viability check.

**Fig. 2.** Incubation plate layout (96-well, clear polypropylene, round bottom, with lid).




**Calibration Curve**

| Conc. (nM) | Pk Area |
|---|---|
| 0 | 6796 |
| 50 | 9122 |
| 100 | 10274 |
| 250 | 13045 |
| 500 | 18338 |
| 750 | 22437 |
| 1000 | 26190 |

| Equation | y=mx + n |
|---|---|
| slope (m) | 18.9 |
| intercept (n) | 8001.9 |

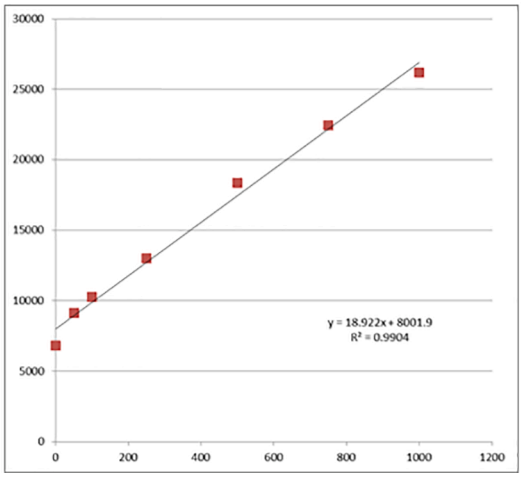



**Fig. 3.** Example MS chromatogram and calibration curve of OCTA: group A (C8) with the corresponding labelled internal standard (13C4-OCTA).

## 2. Method

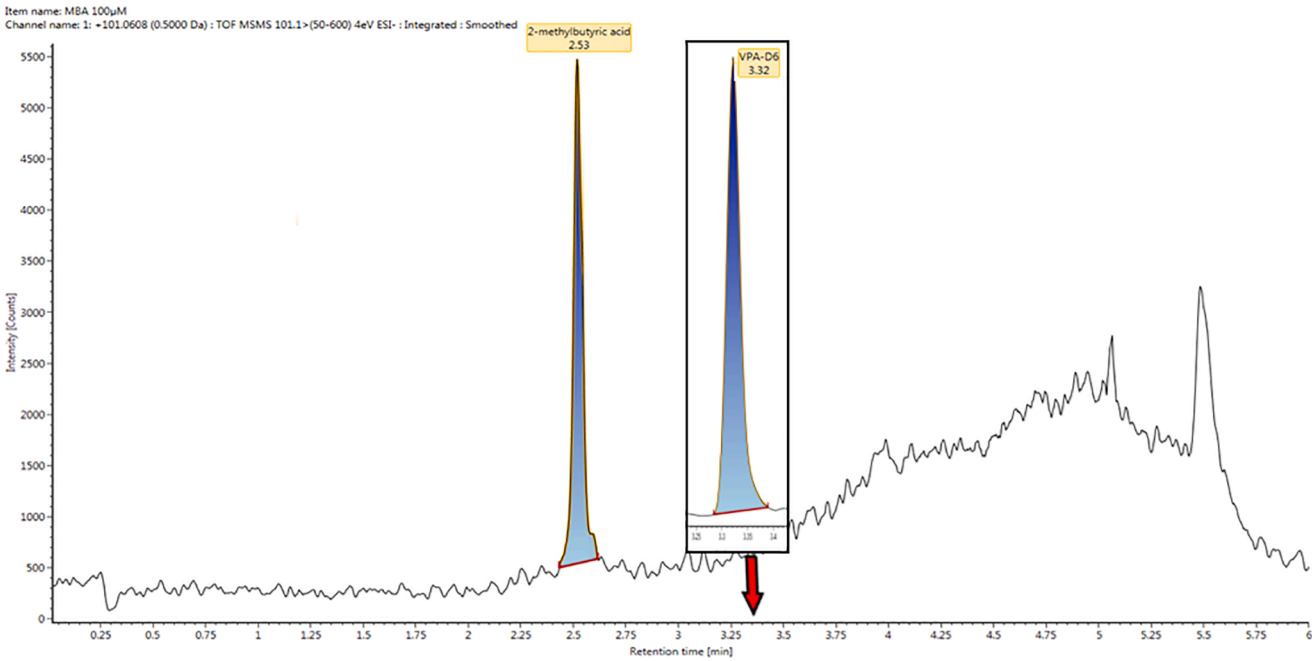
### 2.1. Assay principle

Hepatocytes (cells derived from liver tissue) contain the full complement of metabolising enzymes, maintained within the intact cells. Hepatocytes therefore provide an *in vitro* model for predicting *in vivo* metabolic clearance. Essentially, the hepatocytes were exposed to the test chemicals by incubation in culture medium, preparing replicate samples to enable the interaction to be terminated by addition of a cytotoxic stop solution after appropriate intervals, from time zero to two hours (0, 15, 30, 60, 90, 120 min). Determination of the supernatant chemical concentration with time allows calculation of any depletion rate occurring due to metabolism and/or other biotransformation processes. Physiologically, the clearance of an eliminating organ (e.g., liver) is the volume of blood cleared of xenobiotic (e.g., drug) by that organ per unit of time. By analogy for *in vitro* hepatocytes, the principal conventional measurement is intrinsic clearance $CL_{int}$ (or CL) expressed in μL/min/million cells.

The experimental design (Fig. 1) and incubation plate layout (Fig. 2) illustrate the practical procedure, described in the following sections. In particular, chromatographic profiles and calibration curves of the analytical method are reported in the corresponding graphics (see Figs. 3 and 4).

### 2.2. Primary human hepatocytes in suspension

Primary pooled cryopreserved human hepatocytes (n = 10 donors, lot TQJ, BioIVT) were thawed and washed according to vendor protocol. Hepatocytes were thawed and diluted in 50 mL of InVitroGRO thawing medium and subsequently centrifuged at 50g for 5 min. Cells were reconstituted in warm incubation medium consisting of William's E medium containing Insulin, Transferrin, Selenium (ITS from Gibco cat# 41400045), Hepes (Gibco cat# 15630080) and L-glutamine (Gibco cat# 25030149). Cell number and viability were determined by the trypan blue exclusion method. Cells were suspended to a concentration of $1 \times 10^6$ viable cells/mL in incubation medium. Hepatocytes suspension was only distributed into the three upper rows (R1, R2 and R3) on the left side of six round-bottom 96-well plates at a density of $5 \times 10^4$ cells/well, by adding 50 μL of cell suspension/well. The following lower rows were




**Calibration Curve**

| Conc. (µM) | Pk Area |
|---|---|
| 0 | 988 |
| 5 | 2480 |
| 10 | 4477 |
| 25 | 9828 |
| 50 | 19203 |
| 75 | 28493 |
| 100 | 38052 |

| Equation | y=mx + n |
|---|---|
| slope (m) | 371.6 |
| intercept (n) | 720.1 |

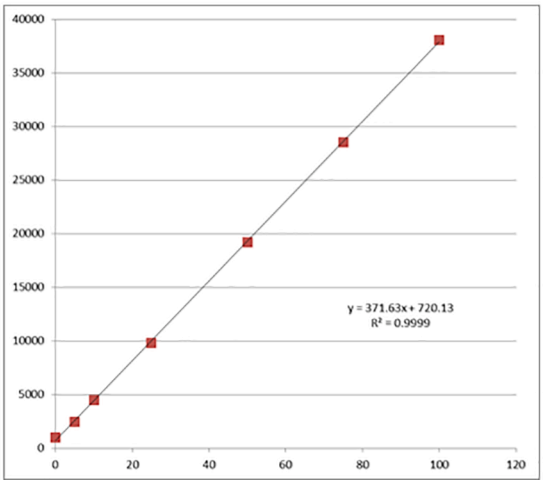



**Fig. 4.** Example MS chromatogram and calibration curve of MBA: group B (C5) with the corresponding labelled internal standard (VPA-D6).

filled with 50 µL of incubation medium without cells to be used as negative control (NC1, NC2 and NC3). In both cases other 50 µL of test item solution/well were added. One plate was used for each time point.

### 2.3. Test items

The six structural analogue test items (Table 1) comprised three with aliphatic carbon composition C8 (group A) and three with C5 (group B). Taking account of analytical sensitivity, hepatocyte exposure was set at 1 µM for group A and 100 µM for group B, also confirmed in advance to be non-cytotoxic. Thus, respective stock solutions were prepared at 1 mM and 100 mM in acetonitrile. Then, for hepatocyte exposure, prescribed by addition of 50 µL test item aliquots to 50 µL cell culture suspensions (incubated on a plate) dilutions to 2 µM and 200 µM in assay medium were first arranged, for 1 µM and 100 µM effective concentrations, with the acetonitrile at 0.1% for cell viability.

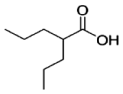
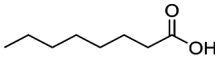
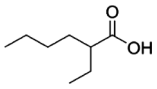
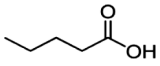
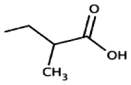
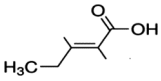
### 2.4. Preparation of samples

The six plates were pre-incubated for approximately 15 min under shaking (200 rpm) in a humidified chamber at 37 °C, with 5 %$CO_2$. The reaction was initiated by the addition of 50 µL/well of each the test compounds prepared at 2 µM or 200 µM in incubation medium for TI1, TI2, TI3 and TI4, TI5, TI6 respectively, as shown in Fig. 2 (yellow area). The total volume reached per well was 100 µL, giving a final test compound concentration of 1 µM or 100 µM and 5 × $10^4$ cells.

Plates were temporarily placed on a heating plate with pre-set temperature (37 °C) while the test compounds were added. They were then returned under the same shaking and incubation conditions, except for the one destined for the time point 0, which reaction was immediately inactivated.

Samples evenly distributed over 120 min were withdrawn during the shaking incubation from each well, at each of the following remaining time points: 15, 30, 60, 90 and 120. Then, for each time point, 70 µL of the incubated hepatocytes suspension were collected from each well, and transferred into a new labelled wells, on the right side of the same plate, containing already 70 µL of ice-cold acetonitrile, including the internal standard at 500 nM of concentration, in order to inactivate the cells. The plates were then centrifuged for 10 min at 4 °C and 1300g. Finally, 100 µL of clear aliquots of supernatant were transferred in

**Table 1**
Test items: short chain fatty acids analogues.

| Chemical name/ Synonym/ ACRONYM | CAS number | M.W. (g/ mol) | No. of C | TI No. | Molecular structure |
|---|---|---|---|---|---|
| Valproic acid VPA | 99-66-1 | 144.1 | $C_8$ | 1 |  |
| Octanoic acid Caprylic acid OCTA | 124-07-2 | 144.1 | $C_8$ | 2 |  |
| 2-Ethyl hexanoic acid 2-Ethyl caproic acid ET-CAPRO | 149-57-5 | 144.1 | $C_8$ | 3 |  |
| Pentanoic acid Valeric acid VLA | 109-52-4 | 102.1 | $C_5$ | 4 |  |
| 2-Methyl butyric acid MBA | 116-53-0 | 102.1 | $C_5$ | 5 |  |
| *trans*-2-Pentenoic acid TPA | 13991-37-2 | 100.1 | $C_5$ | 6 |  |

Chemicals obtained from Sigma-Aldrich (Merck).

**Table 2**
Binary pump steps for chromatographic elution of valproic acid and analogues.

| Time (min) | Mobile phase [a] | | Mobile phase [b] | |
|---|---|---|---|---|
| | A(%) | B(%) | A(%) | B(%) |
| 0.00 | 98.0 | 2.0 | 98.0 | 2.0 |
| 1.50 | 98.0 | 2.0 | 98.0 | 2.0 |
| 4.00 | 30.0 | 70.0 | 15.0 | 85.0 |
| 4.10 | 10.0 | 90.0 | 15.0 | 85.0 |
| 5.00 | 10.0 | 90.0 | 5.0 | 95.0 |
| 5.10 | 98.0 | 2.0 | 98.0 | 2.0 |
| 6.00 | 98.0 | 2.0 | 98.0 | 2.0 |

(a) Program for Group A ($C_8$); (b): program for Group B ($C_5$).

labelled glass vials and kept at low temperature until quantification.

### 2.5. Standard calibration

Quantitative sample analysis was completed by UPLC (Ultra Performance Liquid Chromatography) coupled to a tandem-quadrupole mass spectrometer (Acquity Xevo G2-S QTOF, Waters Co., USA).

Concentration standard curves were generated for group A (0–1000 nM) and group B (0–100 μM) prepared as two dilution series with composition corresponding to the assay analytical mix, including stop solution containing internal standard. An internal standard is a similar molecule with comparable chemical properties, allowing for correction of variability due to analyte loss during sample treatment. Two isotopically labelled internal standards were used, octanoic acid, $^{13}C_4$-OCTA (CAS# 159118-65-7), for chain molecular structure chemicals and valproic acid, VPA-$D_6$ (CAS# 87745-18-4), for branched molecular structure compounds.

**Table 3**
Retention times: test items (TI) and their corresponding internal standards (IS).

| Retention times TI / IS (min) | | | | | |
|---|---|---|---|---|---|
| VPA / VPA-$D_6$ | OCTA/$^{13}C_4$-OCTA | ET-CAPRO / VPA-$D_6$ | VLA / $^{13}C_4$-OCTA | MBA / VPA-$D_6$ | TPA / VPA-$D_6$ |
| 3.57 / 3.57 | 3.67 / 3.67 | 3.56 / 3.56 | 2.57 / 3.41 | 2.53 / 3.32 | 2.41 / 3.32 |

**Table 4**
Electro Spray Ion source settings for test items and internal standards.

| **Source Parameters (MS-QTOF)** |
|---|
| **Common:** ESI polarity **negative**; Source temperature **120 °C**; Desolvation temperature **400 °C** |
| Cone gas flow **30L/h**; Desolvation gas flow **900L/h**; Collision energy **4.00 eV** |
| **Specific:** [voltages capillary(kV)/Cone(V)]: VPA: **2.0/15**; OCTA: **1.0/10**; ET-CAPRO: **1.5/10** VLA,MBA and TPA: **2.5/15** |
| **Mass settings (TI):** VPA(*) OCTA(**) ET-CAPRO(*) VLA(**) MBA(*) TPA(*) (*m/z*) 143.1 143.1 143.1 101.1 101.1 99.1 |
| **Mass settings (IS):** (*)VPA-$D_6$ (**)$^{13}C_4$-OCTA (*m/z*) 149.1 147.1 |

(*) IS for branched molecular structure test items (**) IS for chain molecular structure test items.

**Table 5**
LOD[(a)] and LOQ[(a)] with SD and RSD of blanks and replicates.

| Parameter | GROUP A ($C_8$) | | | GROUP B ($C_5$) | | |
|---|---|---|---|---|---|---|
| | VPA | OCTA | ET-CAPRO | OCTA | MBA | TPA |
| $SD_{blanks}$ (μM) | 0.1 | 0.6 | 0.1 | 67.6 | 145.4 | 48.5 |
| **$RSD_{blanks}$ (%)** | **8.9** | **8.8** | **15.2** | **9.8** | **14.6** | **16.4** |
| LOQ (μM) | 0.04 | 0.23 | 0.05 | 3.0 | 6.0 | 6.0 |
| LOD (μM) | 0.01 | 0.07 | 0.02 | 1.0 | 2.0 | 2.0 |
| **RSD (%) (over all samples)** | **4.8** | **5.2** | **8.4** | **4.3** | **4.8** | **4.5** |

Note: data resulting from the average of the 3 experiments in triplicate.
RSD acceptance criteria < 20%.
(a) calculated according to the "Validation of Analytical Procedures", ICH - Harmonised Tripartite Guideline.

### 2.6. Sample analysis

The UPLC system comprised a binary solvent delivery manager coupled to a BEH C18 column (50x2.1 mm with 1.7 μm particle size packing) maintained at 50 °C. The mobile phase was 10 mM aqueous ammonium acetate buffer (solvent A) and 100% acetonitrile (solvent B) both containing 0.1% acetic acid, delivered at 0.50 mL/min flow rate with programmed concentration gradients (Table 2). The reagents were LC grade, with ultrapure water (18.2MΩ.cm) used for the aqueous solutions, freshly prepared and degassed by ultrasonic immersion. All samples were analysed in triplicate, covering all replicates, negatives controls and QCs, and the volume of each injection was 10 μL.

Observed retention times for the test items (TI) and corresponding internal standards (IS) under the chromatographic conditions (Table 3) were used for sample peak identification.

The MS set-up (Table 4) consisted in the electrospray ionisation (ESI), with source parameters optimised in negative mode. VPA, analogues and IS were detected in multiple reaction monitoring (MRM) mode, using the most intensive mass-to-charge (*m/z*) transitions for quantification. Argon was used as collision gas and nitrogen as nebulizer/desolvation carrier. Collision energy (CE) was set at 4.0 eV and applied to all analytes.

An analysis routine was arranged for each series of test items (sample replicates and negative controls) including standard curves and quality

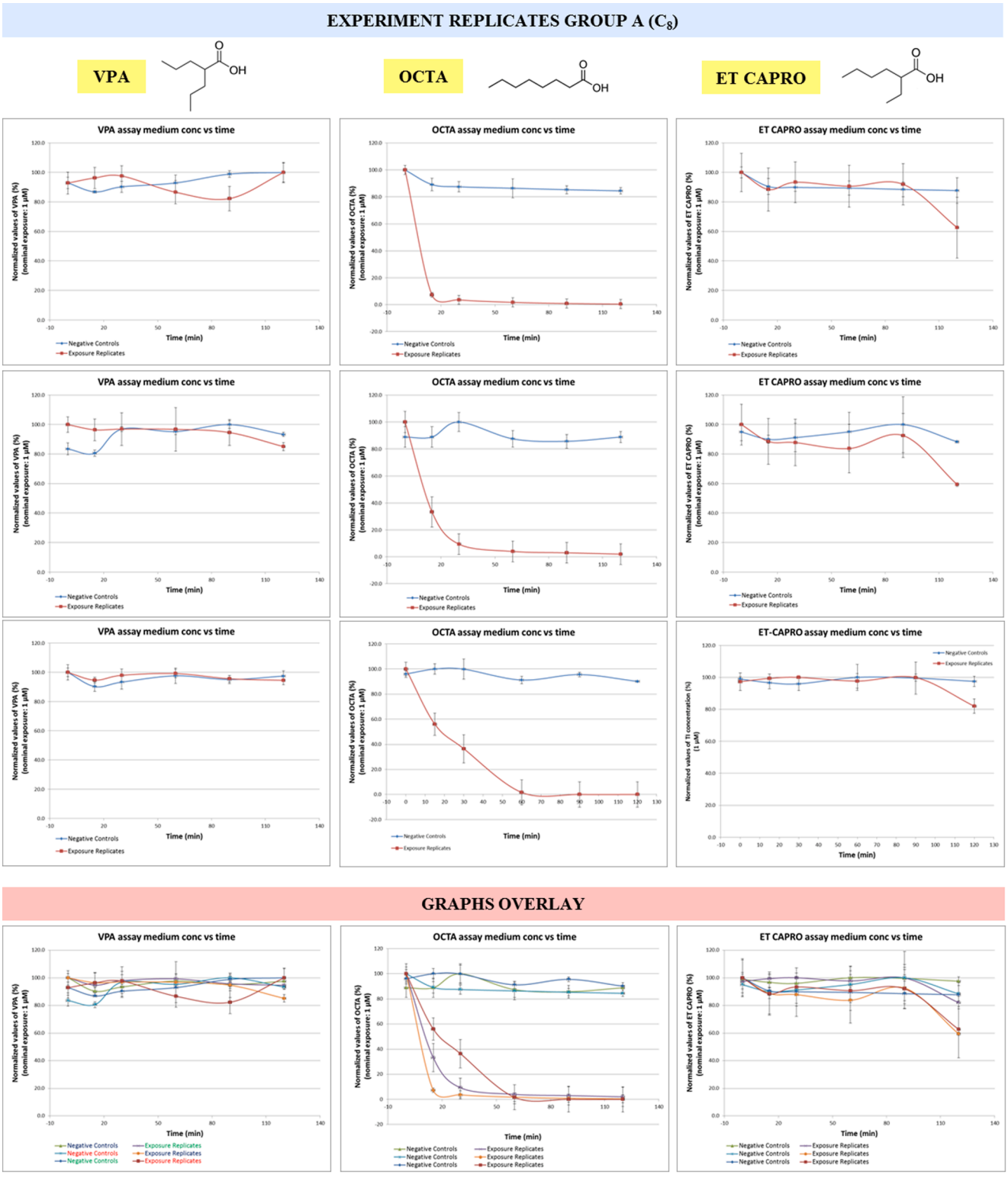



**Fig. 5.** Group A: supernatant concentration vs exposure time (2 h).

controls (QC). Regular insertion of blank column washes ensured absence of possible carry-over. For group A, triplicate QC fixed at 500 nM gave 492.1 ± 30.4 nM with relative standard deviation (RSD) of 6.2%. For group B, triplicate QC fixed at 50 µM gave 49.7 ± 3.2 µM with RSD of 6.4%.

The limits of quantification and detection (Table 5) were calculated from blank peak area standard deviation (SD) and linear slope fit according to convention (*):

$$LOQ = 10*SD_{blanks}/slope$$
$$LOD = 3*SD_{blanks}/slope$$

(*) Validation of Analytical Procedures (ICH HARMONISED TRIPARTITE GUIDELINE)

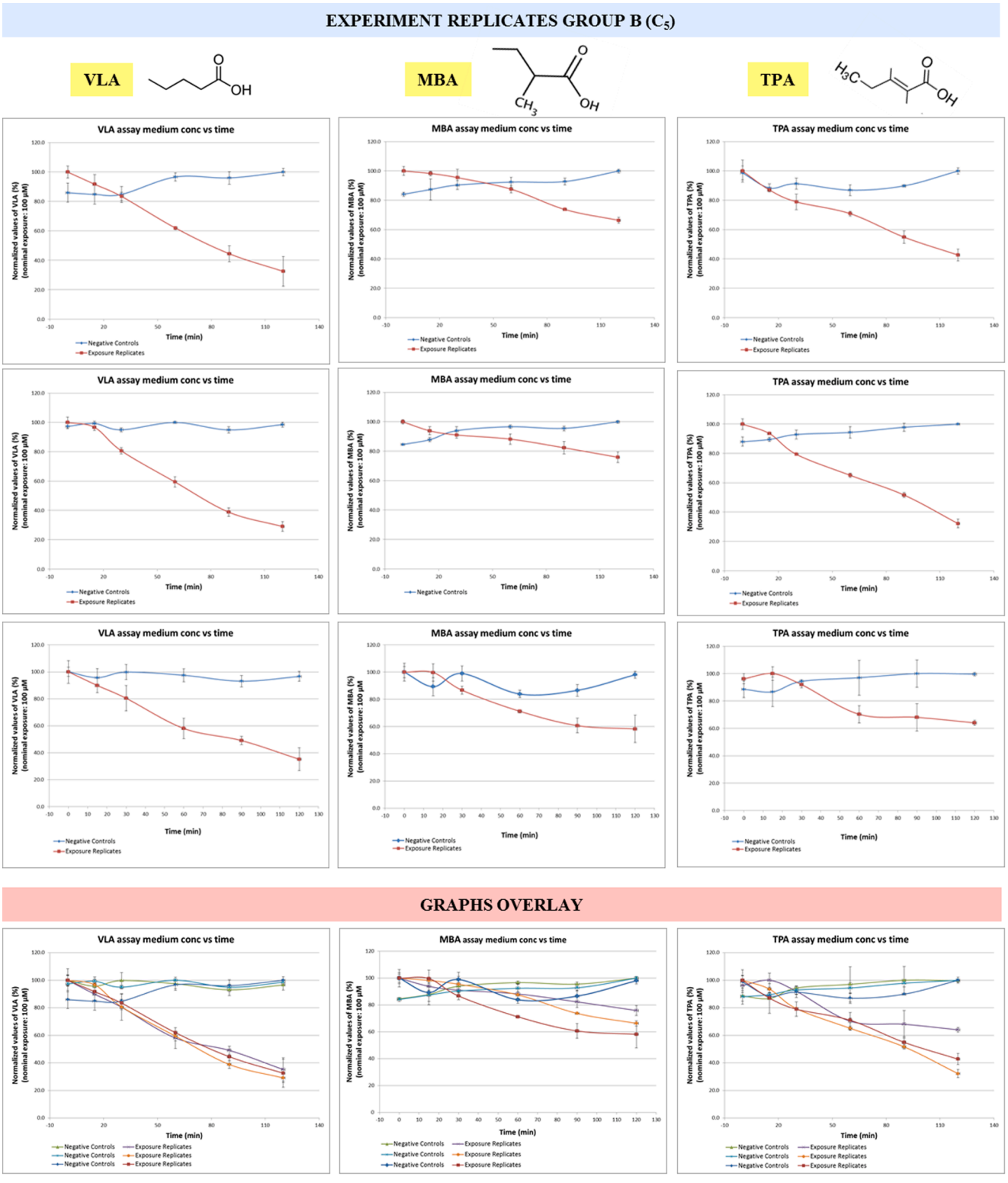



**Fig. 6.** Group B: supernatant concentration vs exposure time (2 h).

### 2.7. Intrinsic clearance

The *in vitro* hepatocyte metabolism was calculated as intrinsic clearance ($CL_{int}$):

$$CLint = \frac{kV}{N} \text{expressedin} \mu L/(min*10^6 cells)$$

where:

k = the elimination rate constant

V = incubation volume (100 µL)

N = number of cells ($x10^6$) incubated (50000 cells = 0.05 million cells)

and according to first order kinetics, k is the linear gradient of $\ln(C_t/C_0)$ vs t(min) applied with 1/y weighting:

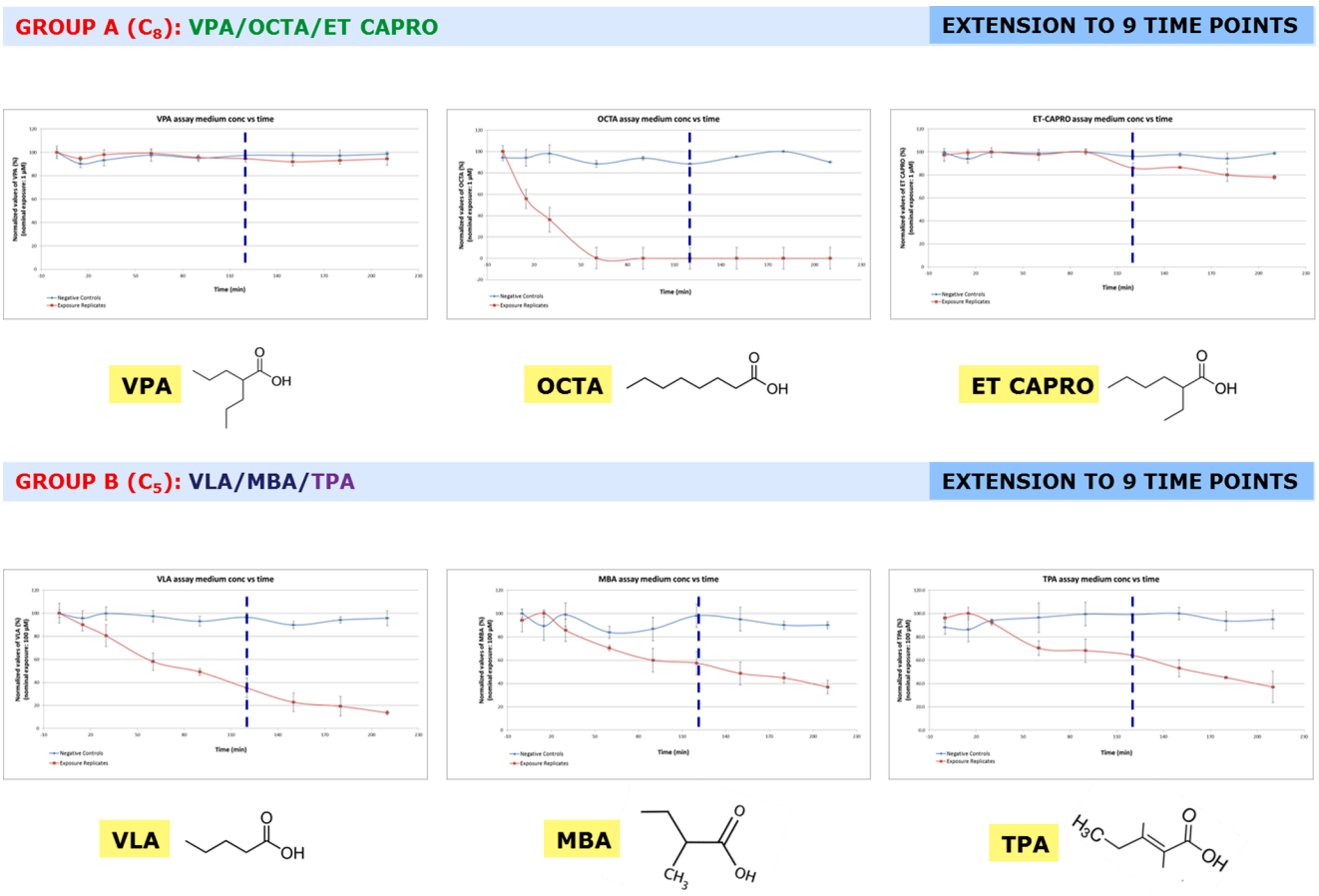



**Fig. 7.** Groups A and B: supernatant concentration vs exposure time (3½ hours).

**Table 6**
Intrinsic clearance values ($CL_{int}$) of the test items and final concentrations in the supernatant fraction after 2 and 3½h of exposure.

| Test Item | $CL_{int}$ (μL/min/$10^6$cells) | Remaining conc. in the supernatant fraction after **2 h of exposure** [(a)] | Remaining conc. in the supernatant fraction after **3½h of exposure** [(a)] | M.W. | No. of carbons |
|---|---|---|---|---|---|
| VPA | 0.9 | 97 | 94 | 144.1 | $C_8$ |
| ET-CAPRO | 4.0 | 68 | 65 | 144.1 | $C_8$ |
| MBA | 7.7 | 67 | 42 | 102.1 | $C_5$ |
| TPA | 12.2 | 46 | 27 | 100.1 | $C_5$ |
| VLA | 19.4 | 32 | 14 | 102.1 | $C_5$ |
| OCTA | 84.4 | 0 | 0 | 144.1 | $C_8$ |

(a) Expressed as % of the initial exposure concentration.

$$\ln\left(\frac{Ct}{C0}\right) = -kt$$

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

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

## 3. Results

Hepatocyte metabolism of the six test item chemicals is shown by plots of supernatant concentration with time (Figs. 5, 6 and 7) respective of group A and B incubated for 2 h, and both groups with exposure extended to 3½ hours. Numerical $CL_{int}$ (Table 6) including remaining concentration fractions (percent) provides a relative order of metabolic

**Table 7a**
Metabolites (known or predicted) for VPA and analogues ($C_8$).

| Valproic acid - VPA | | | |
|---|---|---|---|
| Compound | Molec. Formula | M.W. | *m/z* |
| ***VPA*** | ***$C_8H_{16}O_2$*** | ***144.1*** | ***143.1*** |
| VPA-Glu | $C_{14}H_{24}O_8$ | 320.3 | 319.3 |
| 3-oxo-VPA | $C_8H_{14}O_3$ | 158.2 | 157.2 |
| 3-,4-,5-OH-VPA | $C_8H_{16}O_3$ | 160.2 | 159.2 |
| 2-ene,4-ene-VPA | $C_8H_{14}O_2$ | 142.2 | 141.2 |
| 2-PGA | $C_8H_{14}O_4$ | 174.2 | 173.2 |
| **Octanoic acid - OCTA** | | | |
| Compound | Molec. Formula | M.W. | *m/z* |
| ***OCTA*** | ***$C_8H_{16}O_2$*** | ***144.1*** | ***143.1*** |
| OCTA-Glu | $C_{14}H_{24}O_8$ | 320.3 | 319.3 |
| 7-OH-OCTA | $C_8H_{16}O_3$ | 160.2 | 159.2 |
| 113-fragment | $C_8H_{18}$ | 114.1 | 113.1 |
| Pantothenic acid | $C_9H_{17}NO_5$ | 219.2 | 218.2 |
| **Ethyl caproic acid - ET-CAPRO** | | | |
| Compound | Molec. Formula | M.W. | *m/z* |
| ***ET-CAPRO*** | ***$C_8H_{16}O_2$*** | ***144.1*** | ***143.1*** |
| ET-CAPRO-Glu | $C_{14}H_{24}O_8$ | 320.3 | 319.3 |
| 2-ET-6-OH-Hexanoic acid | $C_8H_{16}O_3$ | 160.2 | 159.2 |
| D-Glucuronic acid | $C_6H_{10}O_7$ | 194.1 | 193.1 |
| ß-OH-Butyric acid | $C_4H_8O_3$ | 104.1 | 103.1 |

**Table 7b**
Metabolites (known or predicted) for VPA and analogues ($C_5$).

| Valeric acid - VLA | | | |
|---|---|---|---|
| Compound | Molec. Formula | M.W. | *m/z* |
| ***VLA*** | ***$C_5H_{10}O_2$*** | ***102.1*** | ***101.1*** |
| VLA-Glu | $C_{11}H_{18}O_8$ | 278.3 | 277.3 |
| 3-OH-Phenyl-VLA | $C_{11}H_{14}O_3$ | 194.1 | 193.1 |
| 2-Butyl-VLA | $C_9H_{18}O_2$ | 158.2 | 157.2 |
| **Methyl butyric acid - MBA** | | | |
| Compound | Molec. Formula | M.W. | *m/z* |
| ***MBA*** | ***$C_5H_{10}O_2$*** | ***102.1*** | ***101.1*** |
| MBA-Glu | $C_{11}H_{18}O_8$ | 278.3 | 277.3 |
| 3-OH-3-MBA | $C_5H_{10}O_3$ | 118.1 | 117.1 |
| 4-OH-MBA | $C_5H_{10}O_3$ | 118.1 | 117.1 |
| Mesaconic acid | $C_5H_6O_4$ | 130.1 | 129.1 |
| **2-trans pentenoic acid - TPA** | | | |
| Compound | Molec. Formula | M.W. | *m/z* |
| ***TPA*** | ***$C_5H_8O_2$*** | ***100.1*** | ***99.1*** |
| TPA-Glu | $C_{11}H_{16}O_8$ | 276.3 | 275.2 |
| Butyric acid | $C_4H_8O_2$ | 88.1 | 87.1 |
| Propionic acid | $C_3H_6O_2$ | 74.1 | 73.1 |

Notes:
- GLU derives from glucuronidation.
- OXO is synonym for KETO and corresponds to the functional group C = O.
- MBA is known also as isovaleric acid; PGA is propylglutaric acid.
- Pantothenic acid is equivalent to B5 vitamin.
- Mesaconic ac. = (2E)-2-Methyl-2-butenedioic acid (one isomeric carboxylic acids from citric acid).
- Butyric ac. = Butanoic acid; Propionic acid = Propanoic acid.

stability.

These results are essentially in line with perceived hepatotoxicity [4] indicated as positive for VPA, ET-CAPRO and TPA (slower metabolism), negative for OCTA and VLA (faster metabolism) and unknown for MBA.

## 4. Discussion

Since years 1940′s, the interest to know, not only the consequences when a chemical interacts on a biological system, but also the fate of such substance in terms of interpreting the toxicological mechanism(s), including detoxification process(es), were of great importance to acquire data as basis of the toxicity and metabolism classification. As example, Weinhouse et al. conducted a study on fatty acid metabolism, in particular octanoic acid, already in 1944 [1].

The chemical selection for this study assumed variable toxicity (potency) dependent on specificity or mode of action [4]. Metabolites of VPA are documented [10,9] and/or expected for all six analogues (Tables 7a and 7b) providing a basis for further investigation and inference for read-across.

Hepatocyte metabolic clearance has become a standard component in TK, relevant to ADME characterisation and chemicals safety assessment, with proposals for establishing an international framework for methodology harmonisation and criteria for performance evaluation [5].

Significantly, the chain molecular structures appeared more labile to biotransformation compared to their branched carbon analogues, with OCTA effectively depleted within about 30 min, but VPA only a few percent at full time.

In the present work, the four requirements needed for a toxicokinetic study were met:

- doses of 1 μM and 100 μM tested proved adequate for chemicals of groups A and B respectively, showing good biological response and viability of the cells over the time
- the *in vitro* system was sensitive enough to give the expected and valid information to be used in the defined target
- the test used is in line with the final objective in reducing the lab animals
- the chosen type of cells is sufficiently representative to compare and to extrapolate the metabolism *in vitro* to *in vivo* one.

In particular, results derived from this study showed that all concentrations values of the NC (negative controls) were in the interval $> 80 > x \geq 100\%$ of the original exposure concentration, and those obtained from the determination of the exposure replicates were reproducible and gave consistent RSDs. It is important to report that in all samples, NCs and Rs, the RSD was below the acceptance value (<20%), passing the established criterion.

Additionally, through the procedure of quantitative determination the QC series showed a constant reproducibility, in terms of signal stability, during the time of analysis.

Other considerations from this work are related to the calibration curves, which in all cases showed $R^2$ values above 0.95 (linear fit). As reported in Table 3, also the IS values gave high reproducibility in relation to the RT and signal intensity, providing a good correction of results.

Table 4 shows that the LOQ, LOD values were compatible with the required sensitivity and suggest the UPLC MS QTOF as a useful analytical instrumental configuration to reach the objectives described for this study.

## 5. Conclusions

In the context of chemical toxicology, and with incentive for research of alternatives to animal *in vivo* procedures, toxicokinetic modelling forms a significant aspect of *in vitro* and *in silico* development. In particular, intrinsic hepatic metabolic clearance has become a standard measure relevant to assessment of physiological response to chemicals (pharmaceuticals, etc.).

All data obtained clearly show good reproducibility and confirm that the test system is suitable for this scope, due to the good response provided.

The use of the UPLC MS QTOF technique demonstrated to have high reliability, with excellent reproducibility and, at the same time, good level of sensitivity.

Results has given also evidence on the influence of structural similarity and clearance, showing difference values of depletion rate of the chemical in the supernatant fraction when cells are exposed to the selected test chemicals.

This study has implemented a laboratory *in vitro* procedure with analytical method for precise determination of intrinsic clearance using valproic acid with five analogues for comparison of the relative reaction between similar structures. Significantly, the chain molecular structures appeared more labile to biotransformation compared to their branched carbon analogues.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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