Electronic Supplementary Material (ESI) for Lab on a Chip.
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Supplementary Information

Exploration and Application of a Liver-on-Chip Device in Combination with Modelling and Simulation for Quantitative Metabolism Studies

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I. Experimental Protocol

CN Bio Liver Chip

Device Set-Up and Priming of the LC-12 Plates

At Day -5, the LC-12 plate was primed with plating medium to avoid dry spots in the channels and to acclimatize the media and the components of the system to the incubator temperature. The LC-12 plates and the PhysioMimix^{TM} MPS Drivers were first wiped with 70% ethanol and afterwards combined. The plating medium was pre-warmed to 37°C and was added (500 µL) to the reservoir side of the reservoir chamber (see Figure S1). The drivers were slid into the PhysioMimix^{TM} Docking Station to run the "Prime" Program, which induces an up-flow of 2.5 µL/s for 3 minutes to the medium in the wells. After this step, the wells were filled with 1100 µL to cover the whole surface of the well with medium. The plate was returned to the docking station to run the "Incubate" program, which induces an up-flow of 2.5 µL/s to medium in the wells until the seeding at Day -4.

Media Exchange

The plates were removed from the incubator and the medium in the wells was aspirated until the remaining dead volume of 200 µL. Then, 400 µL of pre-warmed (37 °C) maintenance medium was added to the wells and the LC-12 plates were returned to the docking station to run the "Media Exchange" program, which induces a down-flow of 1.0 µL/s for 3 minutes. After the 3 minutes, the plates were removed from the docking station and the medium was again aspirated until the remaining dead volume of 200 µL. Then, 1400 µL of plating medium was added to each well and plates were returned to the docking station to run the "Incubate" program.

Seeding of the Cells

At Day -4, the hepatocytes were seeded on the scaffold of the wells in the LC-12 plate. For the process, the cryopreserved hepatocyte recovery medium (CHRM) and plating medium were pre-warmed to 37 °C in the

water bath. Hepatocytes vial(s) were thawed, and the cells were transferred and suspended into 50 mL of CHRM. The cell suspension was centrifuged at room temperature at 100G for 10 minutes, and afterwards, the supernatant was carefully aspirated. Hepatocyte pellet was loosened by gently tapping the falcon tube and re-suspended in 3.0 mL (for two vials of hepatocytes) of plating medium. 50 µL of cell suspension was transferred to 0.1% Trypan Blue for the cell count with the hemocytometer.

The primed plates were removed from the incubator and the medium in the wells was removed as described in the media exchange program, with the difference that the medium added in the last step was not 1400 µL maintenance medium, but 300 µL plating medium in order to prepare the plates for the seeding process. The prepared hepatocyte suspension was now equally distributed to the different scaffolds (100 µL for each well). Note: Important to ensure a well-mixed hepatocyte suspension and to disperse the hepatocytes over the whole scaffold to avoid inter-well variability. Afterwards, the plates were returned to the docking station to run the "Seed" program, which induces a down-flow of 1.0 µL/s for 2 minutes. The plates were removed and 1000 µL of plating medium was very slowly added to the wells to cover the surface of the wells. Finally, plates were returned to the docking station to run the remaining "Seed" program that runs for another 7 hours and 58 minutes and automatically changes to the "Incubate" program afterwards.

Determination of Total Protein

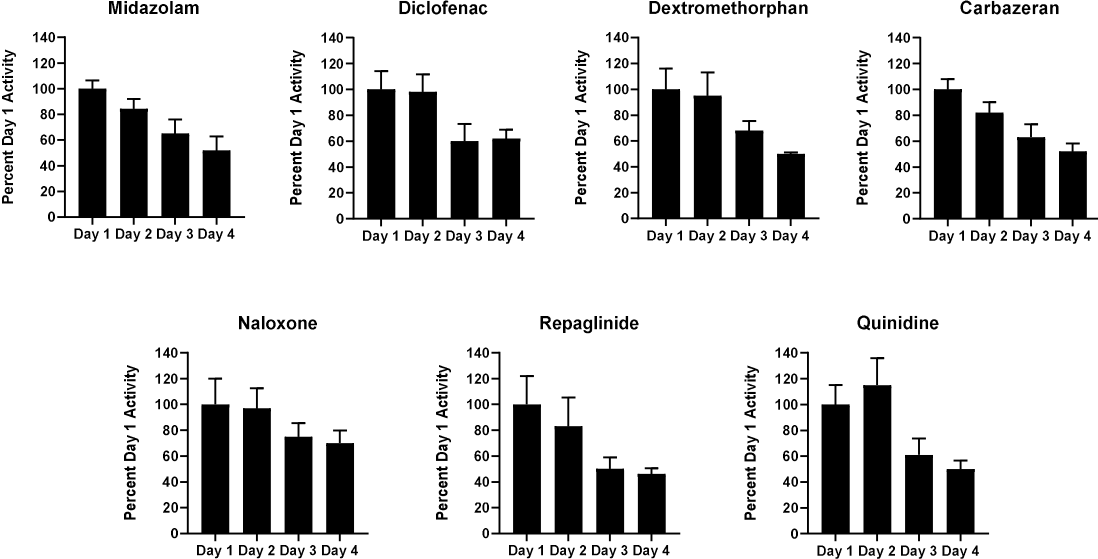
Lysis of the Hepatocytes in the Scaffolds of Control Wells

The scaffolds in the control wells were removed, washed twice in 1000 µL PBS and subsequently placed into 500 µL PBS containing 1% Triton-X. The surface of the scaffold was then thoroughly scratched with a pipette tip to ensure maximal retrieval of contained cells, and the lysing process was continued for half an hour. This process was repeated twice to ensure complete detachment and lysis of the cells. After the scaffold was washed and removed from the cell lysate, total protein content measured with the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific), and the cell number estimated. For the measurements, it was assumed that all attached cells were alive and metabolically active, while dead cells were detached and removed from the scaffold during the washing steps.

II. Figures

Figure S1. Main Liver-on-a-chip components (©CN Bio Ltd., reproduced with permission).

Figure S2. Retention of Drug Metabolism Activity on Repeated System Usage



Analysis were assessed in the Liver-on-a-Chip using different probe substrates over four days. Data are displayed as percent of depletion rate constant relative to Day 1 with the respective standard deviations

Figure S3. Substrate depletion and metabolite formation measurements in the assessment of repeated well use over 4 days.

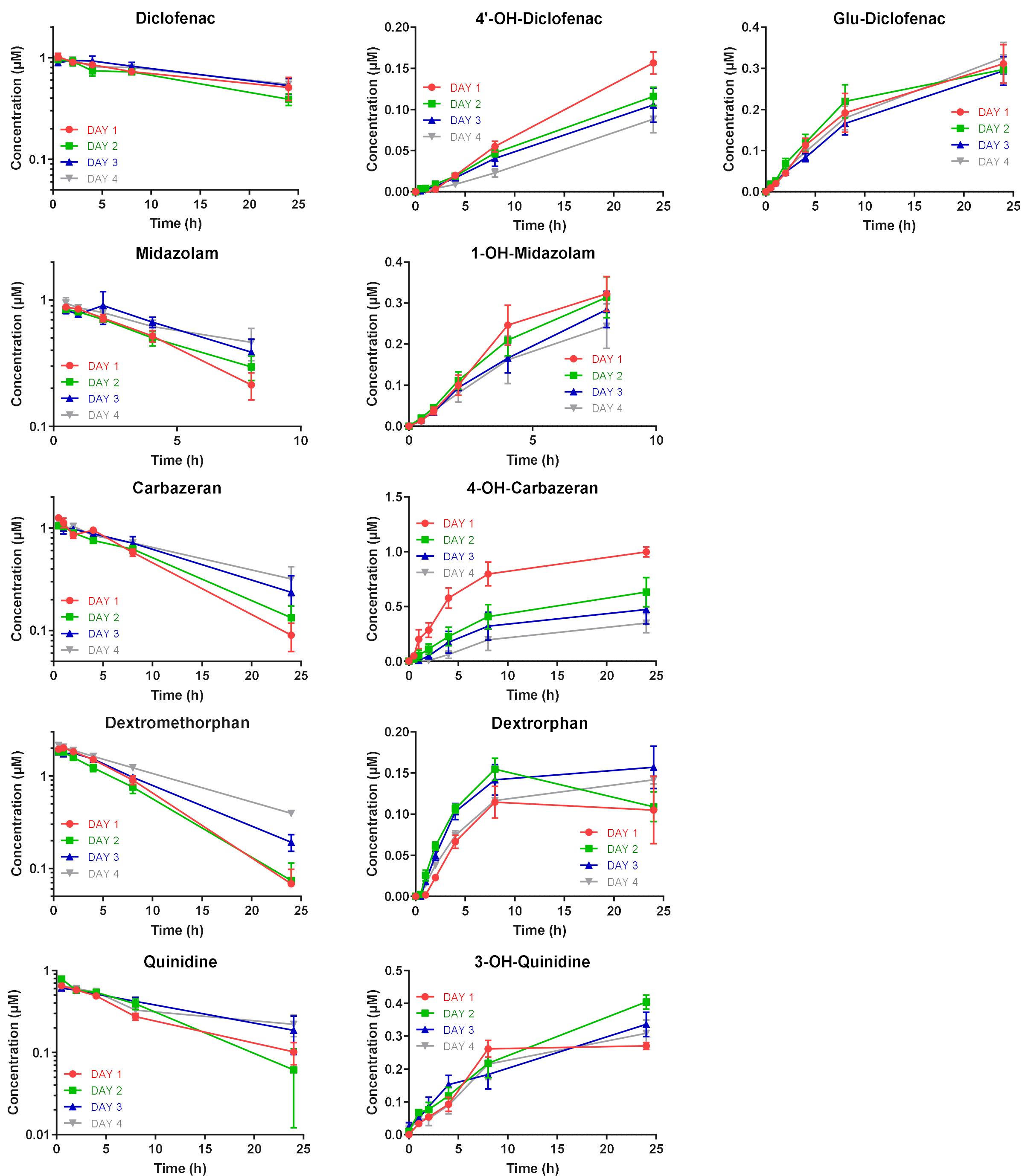
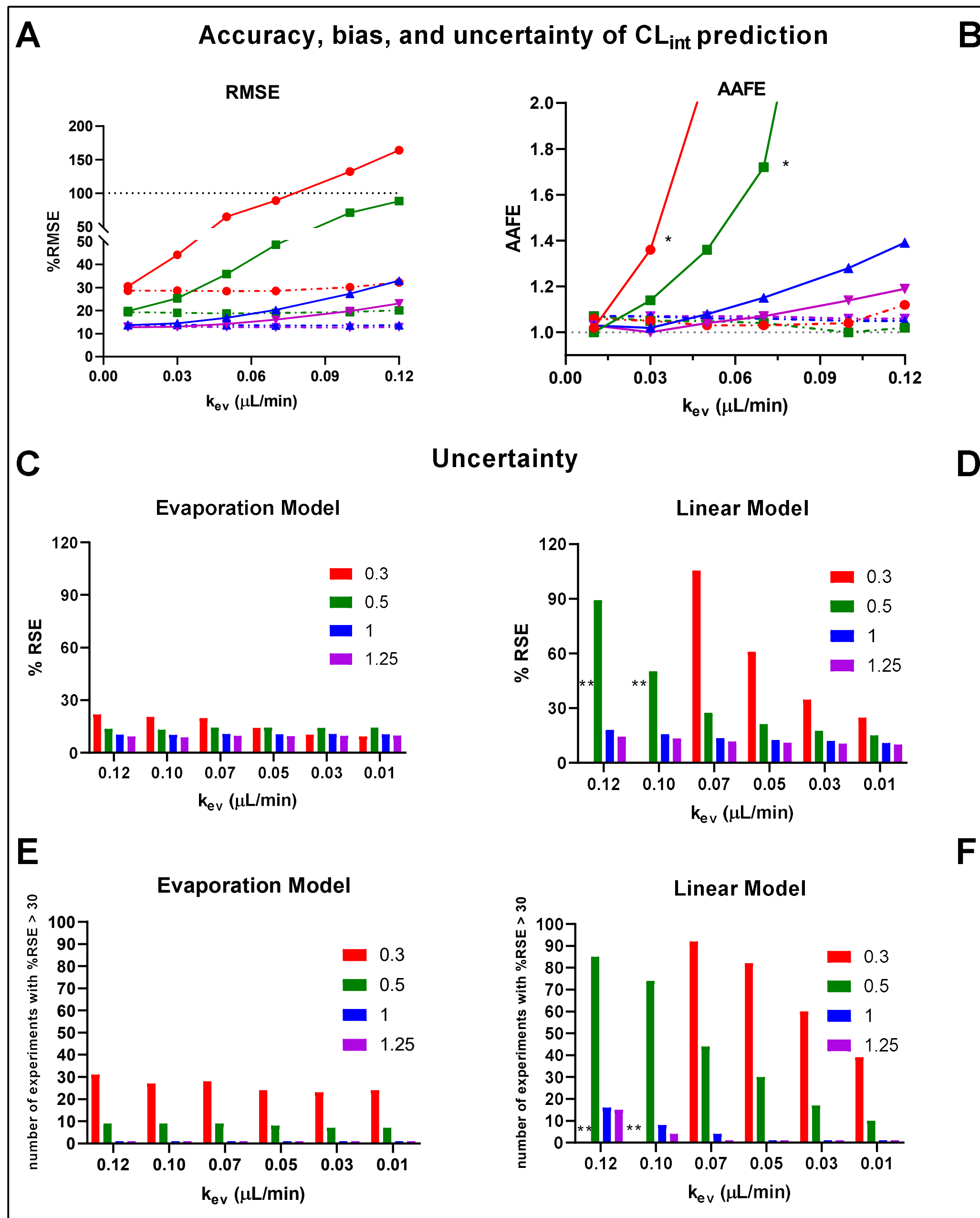


Figure S4. Verification of the linear model and the evaporation model.



Legend

CL_{int} (µL/min/10^6 cells): 0.3, 0.5, 1, 1.25

Model: Linear, Evaporation Model

The graphs report the %RMSE (A), the AAFE (B), C-D), and the uncertainty of CL_{int} estimation from the evaporation model (C) and linear model (D) as function of the k_{ev} for 4 different CL_{int} (0.3, 0.5, 1, 1.25 µL/min/Mio cells in red, green, blue, and purple, respectively). Data were generated by the fitting of 100

simulated experiments in triplicates. %RSE was evaluated by the median of the %RSE of the individual experiments.

From the graph A, a high increase in %RMSE was observed when CL_{int} is below 1 µL/min/Mio cells and $k_{ev} > 0.05$ µL/min. The solid and dot-dashed lines represent the data of the linear model and the evaporation model, respectively.

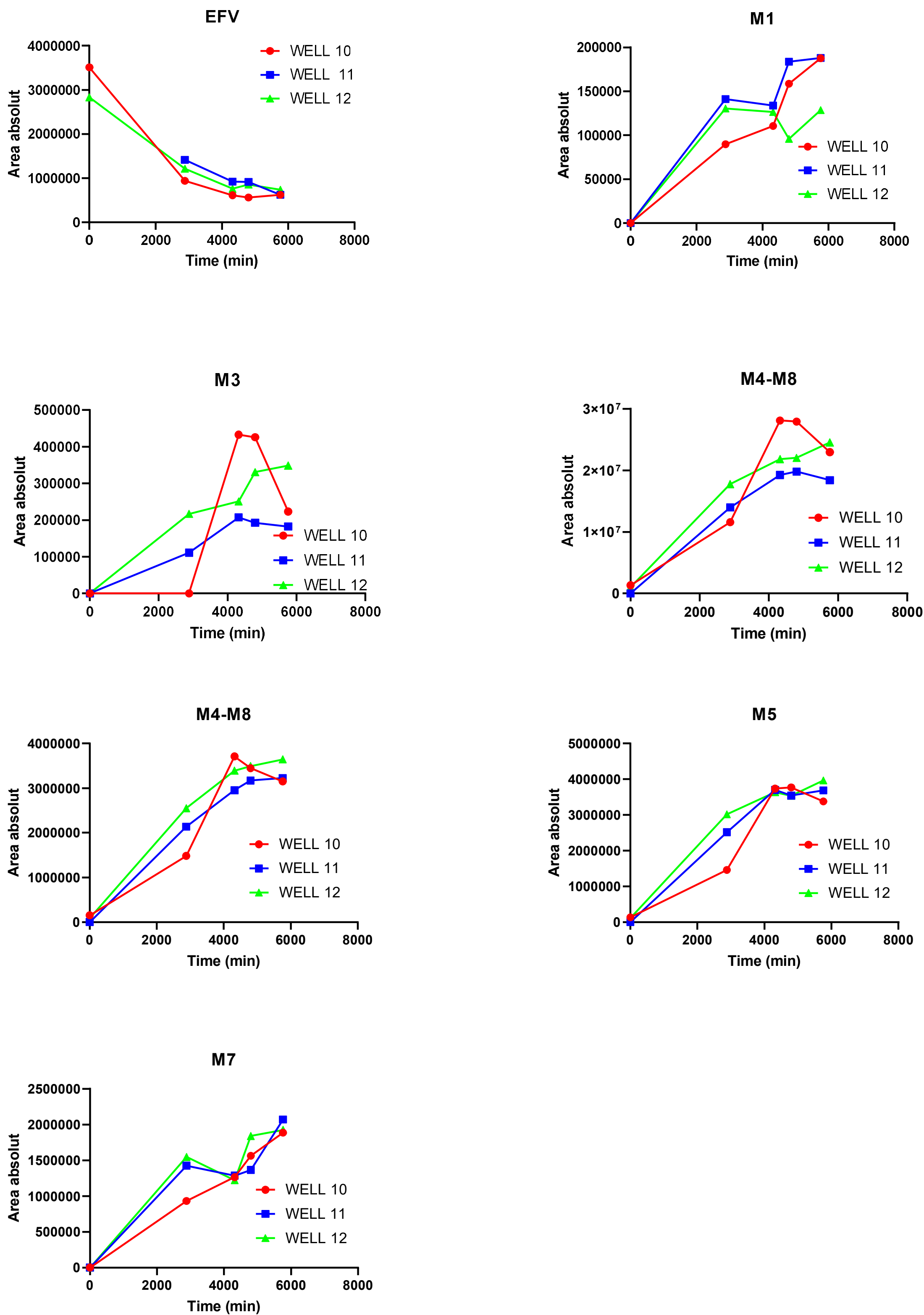
Graph B displays the AAFE as a measure of the bias of the estimation, which is close to unity (dotted line) when the estimation is unbiased. The AAFE increases with decreasing CL_{int} and increasing rate of evaporation. The solid and dot-dashed lines represent the data of the linear and nonlinear model, respectively. The AAFE using the evaporation model was never above 1.08 in all conditions, which denotes low bias of the CL_{int} estimation. * Last point with AAFE < 2.

Graphs C and show the expected CL_{int} uncertainty estimation using the evaporation and the linear model, respectively. Graphs D showed the high uncertainty from the linear model and in particular when k_{ev} more than 0.05 µL/min and CL_{int} below 1 µL/min/10^6 cells.

Graphs E and F reported the number of experiments out of 100 with %RSE more than 30%. There is a high probability (> 30 experiments out of 100) to estimated CL_{int} with high uncertainty (>30%) when k_{ev} more than 0.05 µL/min and CL_{int} below 1 µL/min/10^6 cells with the linear model. On the other hand, the evaporation model provides 31 experiments with %RSE > 30 as the highest value when $k_{ev} = 1.2$ µL/min and $CL_{int} = 0.3$ µL/min/10^6 cells. When $CL_{int} \geq 0.5$ µL/min/10^6 cells the number of experiment with %RSE > 30 was always below 10.

**Median of CL_{int} estimation were < 0 and the bar showing the respective %RSE and number of experiments with %RSE > 30 were not reported.

Figure S5. Data of efavirenz from Liver-on-Chip and the respective primary, secondary, and tertiary metabolites**.**



III. Tables

Table S1: Concentrations, sampling times, and sampling volumes for in vitro intrinsic clearance measurements.

Compounds	Concentration (μM)	Sampling Times (hours)	Sampling Volumes (μL)
Probe Substrates			
Midazolam	1	0.5, 1, 2, 4, 8	30
Dextromethorphan	2	0.5, 1, 2, 4, 8, 24	30
Quinidine	1	0.5, 1, 2, 4, 8, 24	30
Repaglinide	1	0.5, 1, 2, 4, 8, 24	30
Carbazeran	1	0.5, 1, 2, 4, 8, 24	30
Telmisartan	1	0.25, 0.5, 0.70, 1, 2, 4, 8	40
Posaconazole	1	2, 24, 48, 72, 96	18
Naloxone	1	0.5, 0.7, 1, 2, 4	40
Zidovudine	2	2, 8, 24, 32	40
Lorazepam	2	2, 24, 48, 72, 96	40
Efavirenz*	1	2, 48, 72, 80, 96	18
Effect of Evaporation			
Tolbutamide	1	2, 48, 72, 80, 96	18
Irinotecan	1	2, 48, 72, 80, 96	18
Ketoprofen	1	0.5, 24, 48, 72, 96	18
Determination of fm Values			
Oxazepam	2	0.5,1, 2, 6, 8, 24	40
Diclofenac	1	0.5, 2, 4, 8, 24	30

The sampling volume was reduced for metabolically stable compound in order to reduce the impact of the volume depletion due to sampling. For the compounds incubated for 96 h (posaconazole, efavirenz, tolbutamide, irnotecan, and ketoprofen) the initial volume was 1818 μL, whereas 1800 μL was the initial volume for all other compounds.

* Compound tested for semi-quantitative MetID

Table S2. LC-MS/MS methods of the tested compounds

Midazolam, Dextromethorphan, Repaglinide, Telmisartan, Posaconazole, Zidovudine, Lorazepam, Efavirenz, and Diclofenac	
Total flow (mL/min)	0.900
Column	Aeris™ 3.6 µm WIDEPORE XB-C18 (Phenomenex)
Column Temp. (ºC)	50
Injection volume	8 µL in 2 µL loop
Solvent A	Water + 0.1% HCOOH
Solvent B	AcN + 0.1% HCOOH
Gradient program	
Time (min)	% Solvent B
0	5
0.85	98
1.00	98
1.01	5
1.20	5

Quinidine	
Total flow (mL/min)	1.50
Column	Aeris™ 3.6 µm WIDEPORE XB-C18 (Phenomenex)
Column Temp. (ºC)	50
Injection volume	8 µL in 2 µL loop
Solvent A	Water + 0.1% HCOOH
Solvent B	AcN + 0.1% HCOOH
Gradient program	
Time (min)	% Solvent B
0	1
0.90	85
1.23	85
1.30	5
1.33	1
1.70	1

Carbazeran	
Total flow (mL/min)	1.50
Column	Aeris™ 3.6 µm WIDEPORE XB-C18 (Phenomenex)
Column Temp. (ºC)	50
Injection volume	8 µL in 2 µL loop
Solvent A	Water + 0.1% HCOOH
Solvent B	AcN + 0.1% HCOOH
Gradient program	
Time (min)	% Solvent B
0	1
0.15	5
0.75	98
0.90	98
0.91	5
1.20	5

Naloxone	
Total flow (mL/min)	1.50
Column	Aeris™ 3.6 µm WIDEPORE XB-C18 (Phenomenex)
Column Temp. (ºC)	Aeris™ 3.6 µm WIDEPORE XB-C18 (Phenomenex)
Injection volume	8 µL in 2 µL loop
Solvent A	Water + 0.1% HCOOH
Solvent B	AcN + 0.1% HCOOH
Gradient program	
Time (min)	% Solvent B
0	1
0.50	70
0.70	98
0.85	98
0.86	5
1.00	5

Tolbutamide	
Total flow (mL/min)	1.00
Column	Aeris™ 3.6 µm WIDEPORE XB-C18 (Phenomenex)
Column Temp. (ºC)	50
Injection volume	8 µL in 2 µL loop
Solvent A	Water + 0.1% HCOOH
Solvent B	AcN + 0.1% HCOOH
Gradient program	
Time (min)	% Solvent B
0	5
1.00	60
1.50	60
1.51	98
1.80	98
1.81	5
2.00	5

Irinotecan	
Total flow (mL/min)	1.80
Column	Aeris™ 3.6 µm WIDEPORE XB-C18 (Phenomenex)
Column Temp. (ºC)	50
Injection volume	8 µL in 2 µL loop
Solvent A	Water + 0.1% HCOOH
Solvent B	AcN + 0.1% HCOOH
Gradient program	
Time (min)	% Solvent B
0	1
0.85	98
1.00	98
1.01	1
1.20	1

Ketoprofen	
Total flow (mL/min)	0.900
Column	Aeris™ 3.6 µm WIDEPORE XB-C18 (Phenomenex)
Column Temp. (°C)	50
Injection volume	8 µL in 2 µL loop
Solvent A	Water + 0.1% HCOOH
Solvent B	AcN + 0.1% HCOOH
Gradient program	
Time (min)	% Solvent B
0	5
0.85	98
1.00	98
1.01	5
1.20	5

Oxazepam	
Total flow (mL/min)	0.900
Column	Aeris™ 3.6 µm WIDEPORE XB-C18 (Phenomenex)
Column Temp. (°C)	50
Injection volume	8 µL in 2 µL loop
Solvent A	Water + 0.1% HCOOH
Solvent B	AcN + 0.1% HCOOH
Gradient program	
Time (min)	% Solvent B
0	5
0.65	98
0.66	98
0.80	5
1.00	5

Table S3. MS/MS parameters

Compound	Q1 (Da)	Q2 (Da)	DP (volts)	EP (volts)	CE (volts)	CXP (volts)
Midazolam	326.2	291.2	80	10	35	8
1'-OH-Midazolam	342.0	324.0	90	10	31	14
Dextromethorphan	272.2	171.2	111	10	53	16
Dextrorphan	258.2	157.0	100	10	53	14
Quinidine	325.2	184.0	131	10	45	10
3-OH-Quinidine	341.2	172.1	36	10	37	16
Repaglinide	453.2	230.2	70	10	35	8
Carbazeran	361.0	272.1	80	10	30	32
4-OH-Carbazeran	377.0	288.1	80	10	29	12
Telmisartan	515.2	276.2	80	10	65	15
Posaconazole	701.2	683.3	50	10	45	10
Naloxone	328.2	212.1	80	10	52	11
Zidovudine	266.0	223.0	-120	-10	-14	-21
Lorazepam	322.4	277.0	136	10	29	24
Tolbutamide	271.2	91.00	76	10	39	14
Irinotecan	588.2	124.1	151	10	51	10
Efavirenz	315.9	244.1	146	10	19	10
Ketoprofen	255.2	105.1	101	10	33	21
Oxazepam	464.0	288.0	80	10	30	11
Diclofenac	296	214	75	10	40	10
4'-OH-Diclofenac	312.3	230.4	130	10	47	16
Diclofenac-gluc	472.1	214	75	10	40	10

Table S4. Estimation of Low Clearance with the evaporation model and linear model. Values are reported as mean (n = 3) ± SD.

Tolbutamide	**Well 1**	**Well 2**	**Well 3**	$\mathbf{f_{u,med}}$
k_{ev} (µL/min)	0.067	0.057	0.074	
$CL_{int,hep}$ (µL/min/Mio cells) Linear model	0.41 ± 0.06			
$CL_{int,hep}$ (µL/min/Mio cells) Evaporation model	0.59 ± 0.07			
$CL_{int,hep,u}$(µL/min/Mio cells) Evaporation model	1.2 ± 0.1			0.48
Irinotecan	**Well 1**	**Well 2**	**Well 3**	$\mathbf{f_{u,med}}$
k_{ev} (µL/min)	0.057	0.080	0.60	
$CL_{int,hep}$ (µL/min/Mio cells) Linear model	0.41 ± 0.03			
$CL_{int,hep}$ (µL/min/Mio cells) Evaporation model	0.70 ± 0.02			
$CL_{int,hep,u}$ (µL/min/Mio cells) Evaporation model	0.92 ± 0.04			0.76

As additional information about the media evaporation, the k_{ev} of ketoprofen was 0.12 µL/min for all three wells used as triplicates.

Table S5. Compound with the respective source of data for the calculation of the unbound fraction in the medium. The $fu_{inc,pred}$ was also reported and it was calculated as described in the main manuscript.

COMPOUND	$fu_{inc,pred}$	Source	ref.
Posaconazole	0.37	plasma unbound fraction HSA predominantly	1
Dextromethorphan	0.77	K_a in BSA from FTIR	2
Oxazepam	0.60	K_a in HSA	3
Lorazepam	0.80	plasma unbound fraction HSA predominantly	4
Zidovudine	0.98	K_a in HSA	5
Repaglinide	0.36	fu in HSA	6

Sources of data

1. Y. Li, U. Theuretzbacher, C. J. Clancy, M. H. Nguyen and H. Derendorf, *Clinical Pharmacokinetics*, 2010, **49**, 379-396.
2. J. D. Lutz and N. Isoherranen, *Drug Metabolism and Disposition*, 2012, **40**, 159-168.
3. F. D. Boudinot, C. A. Homon, W. J. Jusko and H. W. Ruelius, *Biochemical pharmacology*, 1985, **34**, 2115-2121.
4. P. K. L. Chin, B. P. Jensen, H. S. Larsen and E. J. Begg, *British journal of clinical pharmacology*, 2011, **72**, 985-989.
5. M. A. Quevedo, S. R. Ribone, G. N. Moroni and M. C. Briñón, *Bioorganic & medicinal chemistry*, 2008, **16**, 2779-2790.
6. A. Plum, L. Muller and J. Jansen, *Methods Find Exp Clin Pharmacol*, 2000, **22**, 139-143.

Table S6. Comparison of measured and unbound CL_{int} from LoC, HepatoPac®, and suspended hepatocytes.

	Measured CL_{int} (µL/min/10^6 cells)			Unbound $CL_{int,u}$(µL/min/10^6 cells)		
Compound	**Liver-on-a-Chip**	**HepatoPac**	**Suspended[1]**	**Liver-on-a-Chip**	**HepatoPac**	**Suspended**
Carbazeran	8.7	NA	5.8[1]	12	NA	7.0
Dextromethorphan	16	14[2]	9.6[2]	20	31	11
Diclofenac	4.3	4.3[2]	3.1[2]	95	108	78
Irinotecan	0.70	2.6[2]	0.8[2]	0.92	4.1	0.99
Lorazepam	1.7	1.7[3]	1.6[3] - 0.63[4]	2.1	2.5	2.4 – 0.74
Midazolam	22	44[2]	33[2] - 15[4]	219	174	130 - 16
Naloxone	57	80[3]	35[2] - 60[4]	65	120	52 - 65
Oxazepam	8.6	4.3[3]	3.1[2] - 2.8[4]	14	8.9	6.3 – 3.3
Posaconazole	2.9	3.3[3]	4.7[3]	8.0	21	30
Quinidine	7.5	13[2]	2.6[2] - 4.2[4]	12	17	3.6 - 6.6
Repaglinide	2.0	8.8[2]	3.4[2]	5.5	8.8	18
Telmisartan	9.6	25[3]	3.6[3]	64	161	23
Tolbutamide	0.59	1.3[2]	1.2[2] - 1.1[4]	1.2	4.1	3.7 – 1.3
Zidovudine	1.7	4.3[3]	1.6[3] - 2.4[4]	1.7	7.7	2.9 – 2.4

[1] Data from Wood et al were scaled from predicted in vivo CL_{int} using the well-stirred model reported in the manuscript.

IV. In silico modelling

A priori identifiability study

The priori identifiability study performed with DAISY permits to investigate the global identifiability of nonlinear dynamic models describes by ODEs. This investigation is named a priori because it does not need any kind of experimental data or knowledge of the in vitro system (e.g. concentration vs time profile of a compound, noise of the data). Indeed, this study is purely based on the knowledge of the number of compartments available for sampling and the number of estimated parameters. Therefore, this software does not need any exact input number to provide the identifiability. The input data declared with *LET* can be change without any impact on the final identifiability result.

Model 4

This model considers the metabolism of the substrate (x1), which generates one observed metabolic pathways (x2) and an unobserved pathway. x2 is further metabolized to form an unobserved metabolite.

```
WRITE "- Model 4- "$

% B_ IS THE VARIABLE VECTOR

B_:={u1,x1,x2,y1,y2}$

FOR EACH EL_ IN B_ DO DEPEND EL_,T$

%B1_ IS THE UNKNOWN PARAMETER VECTOR

B1_:={CLh, CLu, CLuM}w$

%NUMBER OF INPUTS

NU_:=1$

%NUMBER OF STATES

NX_:=2$

%NUMBER OF OUTPUTS

NY_:=2$

LET V1 = 1800$

LET fuinc = 0.1$

LET Nhep= 0.3$

%MODEL EQUATIONS

C_:={df(x1,t)=u1-CLh*fuinc*y1*Nhep,

    df(x2,t)=(CLh-CLu)*y1*Nhep*fuinc+CLuM*fuinc*y2*Nhep,

    y1=x1/V1,

    y2=x2/V1}$

FLAG_:=1$
```

DAISY()$

END$

THIS MODEL WAS GLOBALLY IDENTIFIABLE

Model 5

This model considers the metabolism of the substrate (x1), which generates two metabolic pathways (x2 and x3) with both metabolites experimentally detected. The metabolite x2 and x3 are generated by CLh –CLh1 and CLh1, respectively.

```
WRITE " Model 5a"$

% B_ IS THE VARIABLE VECTOR

B_:= {u1,x1,x2,x3,y1,y2,y3}$

FOR EACH EL_ IN B_ DO DEPEND EL_,T$

%B1_ IS THE UNKNOWN PARAMETER VECTOR

B1_:={CLh,CLh1}$

%NUMBER OF INPUTS

NU_:=1$

%NUMBER OF STATES

NX_:=3$

%NUMBER OF OUTPUTS

NY_:=3$

LET V1 = 1800$

LET fuinc = 0.045$

LET Nhep= 0.3$

%MODEL EQUATIONS

C_:={df(x1,t)=u1-CLh*fuinc*y1*Nhep,

   df(x2,t)=(CLh-CLh1)*y2*Nhep*fuinc,

   df(x3,t)=CLh1*y3*Nhep*fuinc,

   y1=x1/V1,

   y2=x2/V1,
```

```
    y3=x3/V1}$

FLAG_:=1$

DAISY()$

END$
```

THIS MODEL WAS GLOBALLY IDENTIFIABLE

Model 6

This model considers the metabolism of the substrate (x1), which generates two detected metabolic pathways (x2 and x3) and additional undetected elimination pathway (CLu). The metabolite x2 and x3 are generated by CLh –CLh1 and CLh1, respectively.

```
WRITE "Model 6a"$

% B_ IS THE VARIABLE VECTOR

B_:={u1,x1,x2,x3,y1,y2,y3}$

FOR EACH EL_ IN B_ DO DEPEND EL_,T$

%B1_ IS THE UNKNOWN PARAMETER VECTOR

B1_:={CLh,CLh1,CLu,CLM}$

%NUMBER OF INPUTS

NU_:=1$

%NUMBER OF STATES

NX_:=3$

%NUMBER OF OUTPUTS

NY_:=3$

LET V1 = 1800$

LET fuinc = 0.045$

LET fuinc2 = 0.045$

LET Nhep= 0.3$

%MODEL EQUATIONS

C_:={df(x1,t)=u1-(CLh+CLu)*fuinc*y1*Nhep,

    df(x2,t)=(CLh-CLh1)*y1*Nhep*fuinc-CLM*y2*Nhep*fuinc2,

    df(x3,t)=CLh1*y1*Nhep*fuinc,

    y1=x1/V1,
```

```
    y2=x2/V1,

    y3=x3/V1}$

FLAG_:=1$

DAISY()$

END$
```

THIS MODEL WAS GLOBALLY IDENTIFIABLE

Model structure coded in R with *RxODE* and *nlminx* library

Simulations of low clearance compounds

```
mod <- RxODE({

d/dt(Vms) = 0   # Sampling volume specified in the event table

Nh = TNh * exp(eta.Nh)   # Random effect of Number of cells

ke = Tke * exp(eta.ke)   # Random effect of Volume deplition for evaporation process (0 order constant)

Vme = Vi - ke * time     # Reduction of Volume for evaporation volume at different time (in the simulation the time starts from
0 to 5760 minutes with step by 1)

V1 = Vme - Vms          # Reduction of volume during the incubation

C1 = A1 / V1            # Conc substrate in the medium

d/dt(A1) = - CLh * fuinc * Nh * C1 # Amount of substrate in the medium (passive diffusion)

f(A1)    = (V1 - Sample.V) / V1      # Remove the certain amount of substrate in the sampling volume at every sampling process

CObs.1 =  C1 *exp(CEps.C1)                # RUV of C1

})
```

V. References

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