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Evaluation of an integrated in vitro–in silico PBPK (physiologically based pharmacokinetic) model to provide estimates of human bioavailability

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Abstract

PK express module is a physiologically based model of first pass metabolism, which integrates in vitro data with an in silico physiologically based pharmacokinetic (PBPK) model to predict human bioavailability (F_H). There are three required inputs: FDp (Fraction dose absorbed, final parameter from iDEA absorption module), protein binding (fu) and disappearance kinetics in human hepatocytes. Caco-2 permeability, aqueous solubility (at multiple pH's), estimated dose and chemical structure are inputs required for the estimation of FDp (Norris et al., 2000; Stoner et al., 2004) and were determined for all compounds in our laboratory or obtained from literature. Protein binding data was collected from literature references and/or Pfizer database. Human hepatocyte data was generated in-house using an automated human hepatocyte method (using Tecan Genesis™ Workstation) as described previously (Reddy et al., 2004). Sixteen compounds (commercial and Pfizer compounds) were chosen to evaluate the PK express model and the bioavailability predicted from the module was compared with known clinical endpoints. For majority of the 16 compounds (approximately 80%), the PK express model F_H values were comparable to the known human bioavailability (F_H) (within 23.7 units of the known human (true) F, except for PF 3, PF 4, PF 6). In conclusion, the PK express model integrates a number of key readily available discovery parameters and provides estimates of human performance by integrating in silico and experimental variables built on a physiological based pharmacokinetic model. Information from this model in conjunction with other ADME data (e.g., P450 inhibition) will enable progression of most promising compounds for further in vivo PK and/or efficacy studies.
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1. Introduction

In the past two decades, there has been increasing applications of various in vitro models in the pharmaceutical industry for the study of absorption, distribution, metabolism and excretion (ADME) (Roberts, 2003). One of the primary reasons for this is due to the fact that unacceptable ADME properties account for 40% of drug candidate failures in the clinical phase of drug development (Roberts, 2003; Prentis et al., 1988). Therefore, it is important to screen for ADME properties of new chemical entities (NCEs) early in the drug discovery/development process with the aim of reducing or eliminating ADME liability in later stages of discovery or development. Recently, with the utilization of combinatorial chemistry, the number of NCE's synthesized has dramatically increased, which results in a shift in demand for earlier implementation of higher throughput in vitro ADME assays (van de Waterbeemd, 2002). Many technologies including automation and bioanalytical advances (liquid chromatography–tandem mass spectroscopy (LC/MS/MS)) have greatly improved the sample throughput for in vitro ADME assays. Consequently, the availability of such a vast amount of in-vitro ADME data (e.g., solubility, permeability, metabolic stability, drug–drug interaction potential, protein-binding, etc.) enables their use in the development of computational models that can be used to screen NCEs and therefore facilitate the optimization of desirable ADME properties to drive drug

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design prospectively (van de Waterbeemd, 2002; Ekins et al., 2000; Krejsa et al., 2003; van de Waterbeemd and Gifford, 2003; Wilson et al., 2003; Li, 2002; Langowski and Long, 2002).

One of the most desirable ADME properties for an NCE is acceptable oral bioavailability (*F*), which is a result of two processes: absorption and first-pass metabolism. In spite of the complexity involved in these processes, attempts have been made to develop computational models to directly predict *F*. One type of the predictive models, Quantitative Structure–Activity Relationship (QSAR) models, involves the superposition of drug-absorption and metabolism processes (Ekins et al., 2000; Krejsa et al., 2003). These models while high throughput in nature provide only low-level information and tend to “bin” molecules without providing guidance on what properties need to be optimized. Another approach is to develop a physiologically based pharmacokinetic (PBPK) model that predicts the kinetics of a drug in human plasma. Recently, Theil et al reported a generic rat PBPK model which utilizes a minimum input of data (in vitro hepatocyte clearance, plasma protein-binding, and lipophilicity) and provides promising results, i.e. <2-fold error between in vitro and in vivo prediction (Theil et al., 2003). The PK express model developed by Lion Bioscience is a physiologically based model of first pass metabolism that provides bioavailability estimates in humans. It requires three inputs: FDp (Fraction dose absorbed, final parameter from iDEA absorption module), protein binding (fu) and disappearance kinetics in human hepatocytes at six concentrations (ranging from 0.5 to 50 μM) and four time points (0, 30, 120, 240 min) and provides an estimate of human oral bioavailability as the output parameter.

In the present study, the PK express model was evaluated for its ability to predict bioavailability and its applicability in drug discovery/development processes. A total of sixteen compounds (including 5 literature compounds and 11 Pfizer compounds) were chosen for this evaluation. A retrospective analysis of the in vitro/in vivo relationship between predicted *F* and known human *F* was conducted in order to assess the utility of this product. Additionally, the predicted *F* was compared with values using parallel tube and well-stirred models as described by Obach (2001).

2. Materials and methods

2.1. Chemicals

Chemicals including tolbutamide, zolpidem, nortriptyline, chlorpromazine, and propafenone were purchased from Sigma (Sigma–Aldrich Co., St. Louis, MO). Leibovitz’s L-15 medium was obtained from Invitrogen Corp. (Grand Island, NY).

2.2. Instrument and materials

For all the human hepatocyte studies a Tecan Genesis 200 Workstation™ Boston (Medford, MA) was employed. This workstation consisted of an eight-channel liquid-handling arm (using standard fixed, non-disposable tips), and was equipped with a six-position 96-well aluminum orbital shaker. Temperatures in the aluminum shaker were maintained at 37 ± 0.1 °C, using a water bath. The LC/MS/MS system consisted of a Sciex API-3000 mass spectrometer from Applied Biosystems (Foster City, CA), two Series 200 Micro Pumps from Perkin-Elmer (Wellesley, MA), a CTC Analytics model HTS-PAL autosampler from Leap Technologies (Carrboro, NC), and a Valco Cheminert 2 position, 6 port switching valve from VICI (Houston, TX). The YMC Basic HPLC columns were from Waters (Milford, MA) and the Lightning Genesis HPLC columns were from Argonaut (Foster City, CA). Ninety-six-well plates were obtained from Beckman.

2.3. Human hepatocytes preparation

Cryopreserved human hepatocytes were purchased from XenoTech, LLC (Lenexa, KS). XenoTech protocol and XenoTech Hepatocyte Isolation Kit (XenoTech LLC, Lenexa, KS) were used for thawing cryopreserved hepatocytes. First, 50 mL of Tube A (media containing Percoll™ solution used in initial cell isolation) and 50 mL of Tube B (media used to wash the isolated hepatocytes) were pre-warmed at 37 °C. Subsequently, cryopreserved human hepatocytes from at least three vials from different lots were removed from the liquid nitrogen freezer and immediately placed in 37 °C water bath for 1.5 min ± 15 s (for 1.5 ml vials) or 2 min ± 15 s (for 4.5 mL vials). Vials of thawed hepatocytes were gently poured into Tube A. The hepatocyte cells were gently re-suspended and centrifuged at room temperature for 5 min at 90 × *g*. The supernatant was discarded and the media in Tube B was added to the cells. The resulting mixture was re-suspended and centrifuged at RT for 3 min at 60 × *g*. The supernatant was discarded and the cells were re-suspended in an appropriate volume of pre-warmed Leibovitz L-15 media. Trypan blue solution in Tube C (from the hepatocyte isolation kit) was used for cell count and viability calculation. Cell viability of >80% was set as criteria in selection of hepatocytes to ensure quality of this assay.

2.4. Automated human hepatocyte assay

In vitro human hepatocyte data for all the compounds were generated using a semi-automated hepatocyte assay (Reddy et al., 2004). Incubations were carried out in 96-well plates using a hepatocyte pool from several individuals at a cell concentration of 0.5 million cells/mL in a total volume of 50 μL. Plates were incubated and shaken at 37 °C for 4 h. Studies were conducted at eight initial concentrations (0.25, 0.5, 1, 2, 5, 10, 25, 50 mM) in singlet with sampling at four time points (0, 30, 120, 240 min). Only six concentrations were used in the PK express module simulation. The resulting samples were analyzed for substrate concentration according to the LC/MS/MS conditions detailed below.

2.5. Bioanalysis/LC/MS/MS conditions

Prior to analysis, 100 μL aliquots of samples were spiked with 10 μL of internal standard (an ethanolic solution of a proprietary Pfizer compound that ionizes in both positive

and negative mode), vortex mixed and then centrifuged at 4000 rpm at 10 °C in an Eppendorf 5810R centrifuge. Aliquots (5 μL, flushed loop injection) of sample were analyzed using a step gradient HPLC method utilizing mobile phase A (water–acetonitrile–formic acid (95:5:0.1, v/v/v)) and mobile phase B (water–acetonitrile–formic acid (10:90:0.1, v/v/v)). Samples were analyzed on a Waters YMC Basic S-5 guard cartridge (2.0 mm × 20 mm, 5 μm particle size). The column was plumbed in line such that the switching valve continually directed aqueous flow to waste and organic flow to the mass spectrometer, switching the column between the two paths. Initially, the samples were loaded onto the column with mobile phase A at 400 μL/min. After a 0.3 min wash step with mobile phase A, the valve switched the column into the path of mobile phase B at 400 μL/min such that it was eluted into the mass spectrometer. At 0.9 min the valve was switched back to its initial position, directing aqueous flow through the column again to allow for re-equilibration. The total run time was 1 min. The mass spectrometer was controlled with Analyst 1.2 software and equipped with a Turbo Ion-spray source. Peak area ratios were calculated by dividing the analyte peak area by the internal standard peak area within Analyst. The following MRM (Multi-Reaction Monitoring) transitions were monitored for the analytes of interest:

PF 1: (+) 297.2 → 160.3; PF 2: (+) 328.3 → 160.3; PF 3: (+) 413.1 → 194.2; PF 4: (+) 330.2 → 122.4; PF 5: (+) 270.9 → 105.3; PF 6: (+) 199.3 → 171.4; PF 7: (+) 559.4 → 440.8; PF 8: (+) 360.2 → 178.3; PF 9: (+) 409.3 → 206.2; PF 10: (+) 452.4 → 344.3; PF 11: (+) 253.9 → 198.4; tolbutamide: (−) 269.2 → 170.1; zolpidem: (+) 307.8 → 235.2; nortriptyline: (+) 264.0 → 117.2; chlorpromazine: (+) 318.8 → 86.1; propafenone: (+) 341.7 → 116.

2.6. FDp

FDp values were either calculated from iDEA Absorption module (Norris et al., 2000; Stoner et al., 2004) or obtained from literature (for commercial compounds) and in-house reports (for Pfizer compounds).

2.7. Protein-binding

For commercial compounds this data was culled from literature (Balant, 1981; Salva and Costa, 1995; Ziegler et al., 1976; Dahl and Strandjord, 1977; Morike and Roden, 1994). For Pfizer compounds, such information was obtained from internal research reports.

2.8. Hepatic bioavailability calculations

To determine the hepatic intrinsic clearance (Cl_{int}), the half-life ($t_{1/2}$) was calculated from a log linear plot of substrate disappearance versus time using WinNonLin (non-compartmental, model 201, version 3.0) and the following equations were employed (Obach, 2001):

$Cl_{int} = (0.693/t_{1/2}) \times$ (g liver/kg body) × (ml incubation/cells incubation) × (cells/g liver) For the calculation of blood clearance ($Cl_{blood} = Cl_b$), several approaches were used to yield optimal results for comparison with PK express module predicted numbers:

$$Cl_b = Q \times (1 - e^{(-Cl_{int}/Q)})$$

(parallel-tube model without including fu)

$$Cl_b = Q \times (1 - e^{(-Cl_{int} \times fu/Q)})$$

(parallel-tube model with fu)

$$Cl_b = (Q \times Cl_{int})/(Q + Cl_{int})$$

(well-stirred model without including fu)

$$Cl_b = (Q \times Cl_{int} \times fu)/(Q + Cl_{int} \times fu)$$

(well-stirred model with fu)

$$E_H = Cl_b/Q$$

$$F_H = Fdp \times (1 - E_H)$$

where Cl_{int} is the intrinsic clearance, Cl_b the blood clearance, Q the liver blood flow, E_H the hepatic extraction. For humans the following values apply: $Q = 20$ mL/min/kg; 21 g liver/kg body; 1.2×10^8 cells/g liver (Obach, 2001).

2.9. PK express model simulation

PK express model is equipped with a data expert that automatically determines the V_{max}, K_m and other parameters that are necessary for simulation. This information along with FDp and fu is then used to calculate F_H. For data simulation, it requires three inputs: FDp (final parameter from iDEA absorption module), protein binding (fu) and disappearance kinetics in human hepatocytes at six concentrations and four time points. For the 16 compounds (Table 1) Fdp values were estimated using iDEA absorption module and listed in Table 2 (Norris et al., 2000; Stoner et al., 2004). In the case of protein binding data, literature and internal reports were used as the reference resources. Compound disappearance kinetics in human hepatocytes were measured in house using the automated human hepatocyte assay with a Tecan Genesis 200 Workstation™ Boston (Medford, MA). After completing the collection of all the required inputs, all the information was manually entered in to the web-based module to provide bioavailability estimation in humans for the compound of interest.

3. Results and discussions

3.1. Literature compounds

Approximately 67 literature compounds were used to train the PK express model. The five literature compounds (Table 1) that were not a part of the training set were chosen for the module evaluation. Overall, the predicted F_H values for the five literature compounds were within two-fold of the known

Table 1
List of model compounds (literature and Pfizer compounds) chosen for the PK express model evaluation

Literature compounds	Known oral %F	Protein-binding (fu) (%)	Literature references
Tolbutamide	80–90	91–96	Balant (1981)
Zolpidem	72 ± 7	92	Salva and Costa (1995)
Nortriptyline	51 ± 5	90–94	Ziegler et al. (1976)
Chlorpromazine	32 ± 19	95–98	Dahl and Strandjord (1977)
Propafenone	5–50	85–95	Morike and Roden (1994)
Pfizer compounds	**Known oral %F[a]**	**Protein-binding (fu) (%)[a]**	**Intended indications**
PF 1	27.0	85	Pain
PF 2	19.5	65	Head trauma/stroke
PF 3	14.3	>99	Anti-psychotic
PF 4	~0	55	Anxiety disorders
PF 5	~0	78	Alzheimer/pain
PF 6	17.4	99.8	Alzheimer
PF 7	65.0	91.8	Heart diseases
PF 8	20	25	Alzheimer
PF 9	55–60	98	Hypertension
PF 10	63	98.68	Hypertension
PF 11	84	67.01	Heart failure

[a] Such information was obtained from Pfizer internal research reports.

clinical oral bioavailability (Table 2). Calculated bioavailability values were higher using well-stirred model than those using parallel tube model. When both the PK express model and the mathematical calculations were compared with human values for their abilities to accurately predict human F_H for the five compounds (with consideration of protein binding information), the PK express model outperformed well-stirred model and parallel tube model for one (propafenone) and two compounds (chlorpromazine and propafenone), respectively, which are low clearance drugs (Table 2). Apparently, incorporation of protein binding in the mathematical calculations slightly increased the calculated values of bioavailability but did not improve the predictive power of these models.

3.2. Pfizer compounds

Eleven Pfizer compounds (Table 1) for multiple indications were selected to evaluate the PK express model. The majority of the compounds (approximately 80%) were predicted by the PK express model to have essentially similar F_H compared to the known human F (except for PF 3, PF 4, PF 6, Table 2). It was known from internal reports that PF 3 had an atypical clearance mechanism due to differences in blood to plasma partitioning, which may have contributed to the discrepancy between predicted and human values. Additionally, it was cleared extensively through non-cytochrome P450 mediated metabolism (aldehyde oxidase). PF 6 demonstrated dose-dependent clearance and bioavailability in preclinical animal

Table 2
All compounds: comparison of literature human oral bioavailability (F) and predicted F_Hs from both mathematical equations (with data measured at 1 μM using parallel tube or well-stirred model with or without protein-binding (PB)) and the PK express model

Compounds	Known oral %F	%Fdp simulated from iDEA Absorption	%F_H using parallel tube model (w/o PB)	%F_H using parallel tube model (w PB)	%F_H using well-stirred model (w/o PB)	%F_H using well-stirred model (w PB)	%F_H from PK express
Tolbutamide	80–90	100	79	80	81	82	62.7
Zolpidem	72 ± 7	100	60	62.5	66.2	68	60.2
Nortriptyline	51 ± 5	88.8	57.7	59.7	62	63.6	41.7
Chlorpromazine	32 ± 19	89.8	16.2	17.2	33.1	33.9	42.8
Propafenone	5–50	94	~0	~0	~0	~0	22.6
PF 1	27.0	100	~0	~0	~0	~0	8.64
PF2	19.5	97.5	34	49.1	47.4	57.8	20.6
PF 3	14.3	79.4	15	15.3	29.8	30	48.9
PF 4	~0	100	22	43.5	39.8	54.6	22.4
PF 5	~0	100	50	58.2	59.1	64.9	0.05
PF 6	17.4	96.8	44.5	44.6	54.5	54.5	63.7
PF 7	65.0	100	57	59.7	64.0	66	60.1
PF 8	20	53.8	34.4	48.1	37.2	48.4	17.5
PF 9	55–60	100	73	73.5	76.1	76.4	64.6
PF 10	63	93	43.7	44.1	53	53.3	59.2
PF 11	84	100	85	89.7	86	91.2	57.4

models and the reason for lack of correction for PF 4 is currently under investigation.

Consistent with previous observations with literature compounds, well-stirred model provided higher bioavailability estimations than parallel tube model did. Bigger differences were seen with compounds that were rapidly cleared in the liver. Similarly, protein binding increased the calculated bioavailability values and the bigger increases were seen with compounds that were poorly protein bond (such as PF8). Additionally, the PK express model performed better (by one compound) in predicting human values when compared to the mathematical models. In the case of mathematical model, predicted F_H's for four compounds (PF 1, PF 4, PF 5), and PF 6 failed to correlate with known human values.

3.3. Assay reproducibility

One of the concerns with respect to any biological assay using cells derived from human tissues is its assay reproducibility due to biological variability and other factors, such as hepatocyte preparation variability and lab-to-lab variability. To minimize the variability due to differences in human hepatocytes, a pool of at least four different lots of human hepatocytes was used to represent an "average" human. For estimation of the effect of other factors on the assay reproducibility, Nortriptyline was run three times on three different days during the module evaluation. Based on the results (Fig. 1) for Nortriptyline, variation of approximately 20% or less was observed (using E_H as the bench marker) for the three measurements (except for one measurement at 10 μM), which is consistent with those observed with similar biological assays internally.

3.4. Detection of non-linear clearance

One of the advantages of the PK express model is its ability to detect non-linear clearance, based on disappearance kinetics in human hepatocytes at multiple concentrations. Such information is valuable to a discovery project team in order to accurately estimate human bioavailability at therapeutically relevant doses. Currently, the commonly adopted human hepatocyte assay in the pharmaceutical industry is to assay the

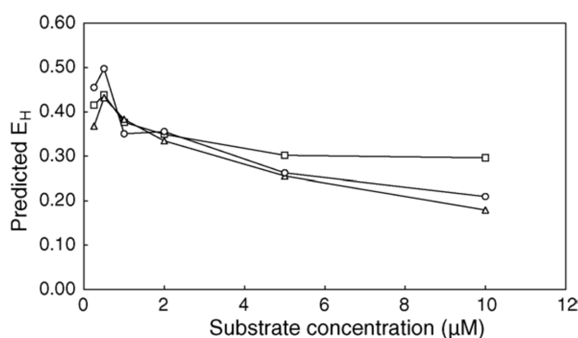

Fig. 1. Assay reproducibility: three individual determination of in vitro human hepatocyte disappearance kinetics for nortriptyline (all data points are mean values of triplicates).

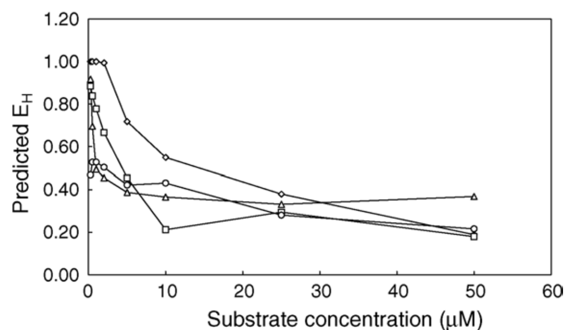

Fig. 2. Examples of dose-dependent clearance for the tested compounds (propafenone diamond; PF 4 square; PF 5 triangle; PF 10 circle) (all data points are mean values of triplicates).

compound at one concentration (generally 1 μM) in hepatocytes and use such information to predict clearance and subsequently bioavailability. The obvious drawback of such approach is that it can result in poor estimations of bioavailability due to unanticipated non-linear clearance. Based on the results obtained in this module evaluation, approximately 40–50% of the compounds tested showed non-linear clearance that needs a range of low to high concentrations to determine. Some of the representative examples of dose-independent and -dependent clearance are listed in Table 3. It is evident (shown in Fig. 2) that rate of disappearance is faster at low substrate concentrations (ranging from 0.25 to 10 μM) than at high concentrations (>10 μM). This phenomenon is likely due to the fact that most of the cytochrome P450 enzymes responsible for the metabolism/clearance of the compounds have K_m values of low μM (Parikh et al., 1997). Therefore, at higher concentration (i.e. >10 μM), compound clearance decreases due to saturation of metabolic processes.

3.5. Module application in early drug discovery

At the early drug discovery stage, information on compound's physical–chemical properties (such as solubility, permeability, and the compound structure) is generated routinely in a high-throughput format, which can be used by iDEA absorption module to provide estimated Fdp values (Norris et al., 2000). Additionally, with the technical advancement in cryopreserving human hepatocytes, human hepatocytes are readily

Table 3
List of examples of tested compounds that showed dose-independent and dose-dependent clearance

Linear compounds	0.25 μM	1 μM	25 μM
Nortriptyline	0.37	0.38	0.25
PF 9	0.21	0.27	0.24
PF 2	0.68	0.65	0.45
Non-linear compounds	**0.25 μM**	**1 μM**	**25 μM**
Propafenone	1	1	0.38
PF 4	0.88	0.78	0.29
PF 5	0.92	0.49	0.33

Listed numbers are calculated mean E_H values of triplicates.

available as lab reagent for routine use. This availability allows for high-throughput assay to be set up to screen hundreds of compounds per week (Reddy et al., 2004). Currently there are internal efforts in Pfizer to develop high-throughput format for protein binding measurements. Such information can also be predicted using in silico tools (van de Waterbeemd and Gifford, 2003). The availability of such vast amounts of in-vitro ADME data (e.g., solubility, permeability, hepatic metabolic stability, protein-binding, etc.) facilitates the use of an integrated PBPK model to provide an estimation of human bioavailability for prioritizing compounds for in vivo PK/efficacy studies. Therefore, the application of the PK express model in the early stages of drug discovery not only provides the team with critical information (human bioavailability estimates) without performing any additional studies, but also helps the team to understand potential non-linear clearance (as previously mentioned), which is of importance for estimating clearance for compounds possessing such properties.

Cautions need to be taken, when applying PK express-estimated bioavailability values in early stages of drug discovery. As it is evident with one of its in vitro inputs, disappearance kinetics in human hepatocytes, PK express will only accurately predict bioavailability where in vivo clearance is predominantly through hepatic metabolism. It is well known that the liver is the major site in the body for xenobiotic metabolism (Roberts et al., 2002). However, tissues, such as intestinal and kidney, can also actively metabolize many xenobiotics (Lohr et al., 1998), In addition clearance can be affected by transporter-mediated influx and efflux (Roberts et al., 2002; Iwatsubo et al., 1991). Therefore, if the clearance of a compound in human is not metabolism related (such as transporter-mediated influx and efflux) or is metabolized via non-hepatic routes, then PK express is expected to underestimate (or overestimate in the case of transporter-mediated influx) in vivo bioavailability.

3.6. Future plans

Preliminary evaluation of the PK express module with 16 compounds (including literature and Pfizer compounds) has provided promising results. Efforts are underway is to select more compounds for further evaluation of this module. The current plan is to take advantage of an external database (BioPrint^{TM} containing clinical information on drugs that are on the market (Krejsa et al., 2003)) and select approximately 50 compounds with known low, medium, high clearance characteristics for a more thorough evaluation of this tool.

One of the inputs for the current PK express model is compound disappearance information in human hepatocytes at six concentrations and four time points, which is not practical in early drug discovery stages. In the Phase II module evaluation, the authors will evaluate an abbreviated algorism that has been trained to use human hepatocyte data at only four concentrations and four time points, which will also decrease the amount of hepatocytes required and hence the number of samples to be analyzed resulting in an overall decrease in resource burden for DMPK labs.

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