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Metabolic stability and its role in the discovery of new chemical entities

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Determination of metabolic profiles of new chemical entities is a key step in the process of drug discovery, since it influences pharmacokinetic characteristics of therapeutic compounds. One of the main challenges of medicinal chemistry is not only to design compounds demonstrating beneficial activity, but also molecules exhibiting favourable pharmacokinetic parameters. Chemical compounds can be divided into those which are metabolized relatively fast and those which undergo slow biotransformation. Rapid biotransformation reduces exposure to the maternal compound and may lead to the generation of active, non-active or toxic metabolites. In contrast, high metabolic stability may promote interactions between drugs and lead to parent compound toxicity. In the present paper, issues of compound metabolic stability will be discussed, with special emphasis on its significance, *in vitro* metabolic stability testing, dilemmas regarding *in vitro-in vivo* extrapolation of the results and some aspects relating to different preclinical species used in *in vitro* metabolic stability assessment of compounds.

Keywords: metabolic stability, biotransformation, intrinsic clearance, *in vitro* half-life, metabolites, new chemical entity

INTRODUCTION

To induce systemic effects in the body, xenobiotics such as drugs must pass plasma membranes in order to bind to the appropriate receptors. Subsequently, active lipophilic compounds are transformed to more polar derivatives, which can be easily eliminated from the body.

In general, drug metabolism can be divided into phase I and phase II. Phase I enzymes are cytochrome P450 (CYP) monooxygenases and flavin containing monooxygenases (FMO), which catalyze oxidation, reduction and hydrolysis reactions. In phase II biotransformation, metabolites rendered in phase I undergo conjugation reactions mediated by enzymes such as UDP-glucuronyl transferases or sulfotransferases (1–5). Determination of metabolic

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stability and fate of a new chemical entity (NCE) is a crucial step in the early phases of the drug discovery and development process, since it greatly influences pharmacokinetic characteristics of therapeutic compounds (6–8). Key issues in this field are drug metabolic stability and drug metabolic profile evaluation.

In general, therapeutic compounds can be divided into those which are metabolized relatively fast and those which undergo slow biotransformation. Rapid biotransformation usually reduces exposure to the bioactive maternal compound and may lead to the generation of active, non-active or toxic metabolites. However, too metabolically stable compounds may promote interactions between drugs and favour parent compound toxic effects.

Nowadays, one of the main challenges of medicinal chemistry and scientists is not only to design chemical compounds demonstrating beneficial activity, but also to select molecules exhibiting suitable pharmacokinetic parameters necessary to elicit a favourable duration of action (9). Therefore, metabolic stability issues of compounds will be discussed in this paper, with special emphasis on the significance of metabolic stability, overview of *in vitro* metabolic stability testing, dilemmas regarding *in vitro-in vivo* extrapolation (IVIVE) of the results and some aspects relating to different preclinical species used in NCE *in vitro* metabolic stability evaluation.

Thus far, several good reviews in the field have been published (2, 10–16). The above mentioned papers, however, were mainly focused on single issues such as *in vitro* metabolic stability testing development, comparison of methods for the assessment of drug metabolic stability, and prediction of *in vivo* metabolic parameters using data from *in vitro* assays.

METABOLIC STABILITY AND ITS SIGNIFICANCE

Metabolic stability refers to compound susceptibility to biotransformation. Both *in vitro* half-life ($t_{1/2}$) and intrinsic clearance (CL_{int}) are utilized to express metabolic stability. $t_{1/2}$ is used to express the time for 50 % disappearance of the parent compound, whereas CL_{int} describes the maximum activity of liver (microsomal proteins or hepatocytes) towards a compound not influenced by other physiological determinants such as hepatic blood flow and drug binding within the blood matrix (2, 17). Further, additional indices including the compound hepatic clearance (CL_H), bioavailability, and *in vivo* half-life can be predicted with different models (2, 18–21).

As most therapeutic compounds are biotransformed in the liver tissue, especially CL_H parameter is of great importance during drug development (16).

When NCE metabolic stability is evaluated with a liver microsomal model, microsomal intrinsic clearance ($CL_{int,micr}$) is determined. $CL_{int,micr}$ (μL min^{-1} mg^{-1}) is then scaled to *in vivo* intrinsic (hepatic) clearance (CL_{int}) (mL min^{-1} kg^{-1}) using suitable scaling factors [including microsomal protein content per gram of liver (MPPGL) and liver mass] obtained from the literature (Table I) (2, 19, 20, 22, 23).

NCE metabolic stability can be interpreted using different approaches, since compounds can be ranked in terms of their CL_{int} and *in vitro* $t_{1/2}$ values or based on parent structure loss during metabolic reactions. According to Di and Obach (31), the low limit of $CL_{int,micr}$ measurements is about 12 μL min^{-1} mg^{-1} protein. McNaney *et al.* (20) classified compounds with CL_{int} above 45 mL min^{-1} kg^{-1} as high clearance compounds, NCE with

Table I. Metabolic stability in liver microsomes, in vitro half-life and intrinsic clearance determination

Symbol	Description	How to calculate/establish	Unit	References
$t_{1/2}$	*In vitro* half-life	Established from the slope of linear regression of the percentage parent compound remaining against time	min	2, 24, 25
$CL_{int,micr}$	Microsomal intrinsic clearance	Based on the equation: $Cl_{int,micr} = \ln 2/t_{1/2} \times$ [volume of incubation medium (μl)/microsomal protein in incubation (mg)]	μL min^{-1} mg^{-1}	2, 24–26
CL_{int}	*In vivo* intrinsic (hepatic) clearance	Estimated from liver microsomal data using the equation and suitable scaling factors: $CL_{int} = CL_{int,micr} \times$ (mg microsome g^{-1} liver) × [liver mass (g)/body mass (kg)]	mL min^{-1} kg^{-1}	26–30
		Scaling factors: 45 mg of microsomal protein per gram of liver tissue (humans, mice, rats, dogs, monkeys/value is applied to all species) 87 g, 40 g, 30 g, 32 g, and 26 g of liver tissue per kilogram of body weight is used for mice, rats, dogs, monkeys, and humans, resp.		

CL_{int} values between 15 and 45 mL min^{-1} kg^{-1} as intermediate clearance compounds, and agents with CL_{int} below 15 mL min^{-1} kg^{-1} as low clearance compounds.

High CL_{int} and the corresponding low *in vitro* $t_{1/2}$ values mean that the compound is rapidly metabolized, and its bioavailability *in vivo* will probably be low (2). Therefore, compounds can be classified on the basis of their $t_{1/2}$ values; *e.g.*, in case of human CYP3A4 supersomes, compounds with $t_{1/2} > 30$ min were classified as long $t_{1/2}$ compounds (*e.g.*, carbamazepine and antipyrine); moderate $t_{1/2}$ agents (*e.g.*, ketoconazole) were characterized with $t_{1/2}$ in the range between 10 and 30 min, and short $t_{1/2}$ compounds (*e.g.*, loperamide and buspirone) were those with $t_{1/2}$ below 10 min (32). On the other hand, in case of the microsomal stability assay with mouse liver microsomes, the following $t_{1/2}$ classification was suggested: compounds with $t_{1/2} \geq 60$ min were defined as being stable in MLM and those with $t_{1/2} < 30$ min were categorized as unstable agents (33).

Moreover, the parent structure loss can be monitored during an appropriate incubation period (*e.g.*, after 15 min) and three categories of substrate depletion can be distinguished: very fast (> 80 %), fast (50–80 %), moderate (20–50 %), slow (5–19 %), and very slow (< 5 %) (34).

Successful drug molecules should achieve and maintain sufficient concentrations at their sites of action and should be removed slowly from the body to ensure appropriate exposure to allow its effectiveness. In this context, the biggest challenges for the pharmaceutical industry during the drug discovery and development process are high clearance values, high metabolic liability and the formation of active or toxic metabolites (35).

It is noteworthy that compounds demonstrating high clearance values are usually immediately removed from the body, which leads to short duration of their therapeutic effect. On the other hand, lower clearance compounds are characterized by reduced doses,

Table II. Some examples of low, intermediate and high clearance drugs (assay in human liver microsomes, HLM)[a]

Intrinsic clearance ($mL\ min^{-1}\ kg^{-1}$)		
Low	Intermediate	High
Quinidine – 3.4	Chlorpromazine – 25.0	Verapamil – 122.0
Clozapine – 4.6	Imipramine – 19.0	Propafenone – 166.0
Dexamethasone – 3.0	Ketamine – 27.0	Midazolam – 160.0
Prednisone – 2.7	Triazolam – 19.0	Diclofenac – 189.0
Diazepam – 2.3		
Zolpidem – 2.8		
Tenoxicam – 1.7		

[a] Ref. 40.

enhanced exposure and prolonged half-life and are hence suitable for once-daily dosing (31, 36–39). Table II provides some examples of drugs in use characterized by low, intermediate or high clearance values.

Today, in the drug discovery process great emphasis is put upon the identification of NCEs with most favourable metabolic profiles (41–43). Therefore, in-depth analysis of the chemical structure of drug candidates and identification of molecule fragments mostly responsible for its biotransformation are conducted. Modification of these "soft spots" provides the opportunity to improve the metabolic characteristics of new chemical compounds (12, 44).

Favourable metabolic stability profiles of NCEs are characterized by prolonged half-life and enhanced bioavailability. Moreover, reduction in metabolic turnover rates from different species is beneficial (35).

Assessment of metabolic stability. – It is vital to conduct metabolic stability studies of NCEs during the early phases of the drug discovery process, since despite showing promising *in vitro* activity, some molecules failed during *in vivo* evaluation due to poor pharmacological and toxicological results (31).

In general, drug metabolism studies are based on *in vitro* cellular/sub-cellular and *in vivo* animal models (14, 36, 37, 45–48). Undoubtedly, studies conducted *in vivo* with animal models are the most valuable source of data regarding NCE absorption, distribution, metabolism and excretion (ADME). Most importantly, results obtained with *in vivo* assays provide a complex pharmacokinetic profile of potential drug candidates. Notwithstanding, tests conducted in living systems are time-consuming, expensive and are not suitable for huge amounts of compounds. Therefore, the most recommended strategy involves preliminary *in vitro* assays that ensure selection of the optimal *in vivo* model to be employed in further drug development stages (35, 49–51).

In vitro metabolic models represent a valuable alternative to animal testing, especially when large libraries of NCEs need to be evaluated and when small quantities of investigated compounds are available. Preliminary *in vitro* data provide the opportunity of targeted synthesis of compounds with favourable metabolic profiles, thus leading to significant cost and time reduction (2, 7, 34, 48).

Metabolic stability testing can be performed by *in vitro* incubation of a compound with suitable competent metabolic models (*e.g.*, liver microsomes, hepatocytes, cDNA-expressed CYP enzymes). Next, chromatographic analysis (*e.g.*, HPLC-MS/MS) of the resulting incubation mixtures is performed (52–54).

Among the routinely used metabolic stability *in vitro* systems are microsomes and hepatocytes. Microsomes are usually used to assess the CYP-mediated phase I metabolism. Some of the most important advantages of using microsomes are accessibility, various species models, procedure simplicity, limited amount of test agents used in the study and the fact that microsmes can be stored for a long period of time. On the other hand, hepatocytes, which contain intact cell membranes and physiological concentrations of enzymes, provide the most physiologically relevant model for predicting hepatic clearance (2, 15, 18, 37, 39, 48, 55–58). Some researchers use microsomes in conjunction with hepatocytes to obtain more comprehensive results (7, 55, 59–61).

Metabolic stability in liver microsomes. – In microsomal stability assays, microsomes coming from different species are used (*e.g.*, human liver microsomes, HLM; mouse liver microsomes, MLM; rat liver microsomes, RLM; monkey liver microsomes, MnLM, or dog liver microsomes, DLM) (62). When human instead of animal based tests are applied, human clinical outcomes can be assessed (16). Interestingly, stability assays with MLM are not considered an ideal model for further *in vivo* experiments with mice. On the other hand, MLM is a good preliminary *in vitro* tool that can correlate well with the results obtained in metabolic assays with HLM (63, 64).

In general, during *in vitro* metabolic stability assays, compounds are incubated with liver microsomes from different species. Chromatographic techniques are then used for metabolite identification (65).

In a typical microsomal stability assay, the incubation mixture consists of a test compound (dissolved in, *e.g.*, acetonitrile, DMSO or methanol), liver microsomes, NADPH-regenerating system, and potassium phosphate buffer. In order to prevent too much non-specific binding, protein concentrations usually do not exceed 2 mg mL^{-1}. In the first step, the mixture containing microsomes, test compound and buffer is pre-incubated at 37 °C for 15 min before addition of the NADPH-regenerating system. Then, the resulting mixture is incubated at 37 °C for several time intervals (*e.g.*, 15, 30 and 60 min). Incubation is usually not longer than 60 min to provide optimal conditions for enzymatic activity. Subsequently, an internal standard is added and the samples are quenched by the addition of chemicals such as perchloric acid, ice-cold methanol or cold acetonitrile. Next, the samples are centrifuged and supernatants are analyzed using chromatography methods to determine the metabolic profile of the test compound. For control samples, the NADPH-regenerating system is replaced by phosphate buffer (2, 31, 66–76).

Metabolic stability in hepatocytes. – In the metabolic assay, hepatocytes coming from different species can be used (*e.g.*, human, mouse, rat and dog). Hepatocyte cultures

represent a complex metabolic system, equipped with natural orientation for linked enzymes, intact cell membranes and all the necessary physiological co-factors (14, 15, 37, 56–59). On the other hand, one of the main concerns about using freshly isolated human hepatocytes for predicting metabolic stability is their longer-term availability and their de-differentiation in culture (16, 31, 39, 48, 77, 78). To solve this problem, cryopreserved hepatocytes can be used (79). Moreover, some new strategies were developed, including highly specialized systems such as HepatoPac or LiverChip. HepatoPac is a micropatterned hepatocyte-fibroblast co-culture system that can be used for continuous incubation of up to 7 days, whereas LiverChip applies to a microfluidically perfused 3D hepatocyte culture system and was found useful in making low clearance measurements (2, 31, 39, 48, 55, 80).

During a standard procedure, hepatocyte suspensions containing 106 cells mL^{-1} are prepared. After preincubation of the cell suspension (10 min, 37 °C, 5 % CO_2), the investigated compound solution (*e.g.*, in DMSO) is added to the cells. The resulting mixture is then incubated again. Samples are taken at different time points (*e.g.*, 15, 30, 60 and 90 min), and reactions are stopped with ice-cold acetonitrile or methanol. Hepatocyte-free control incubations are prepared alongside by spiking a test compound sample into culture media and aliquots are collected at 0 and 90 min. Supernatants are then analyzed using chromatography methods for parent drugs and their metabolites (55, 63–65, 81–83).

Metabolic stability in recombinant expressed enzymes. – Recombinant expressed enzymes provided usually by the baculovirus or the human lymphoblastoid system are the source of specific P450 isoenzymes. Among the main advantages of using the recombinant expressed enzyme model in NCE stability testing are the simplicity of the method and the possibility of using single enzymes for the study. On the other hand, the absence of the remaining phase I and phase II enzymes can be considered as a drawback of this system (2, 15).

In a standard assay procedure, the incubation mixture is composed of a test compound solution, recombinant P450 isoenzymes, potassium phosphate buffer and $MgCl_2$. Firstly, the mixture is pre-incubated for 15 min at 37 °C. Then, NADPH is added to start the metabolic reaction and incubation is continued for different time points (up to 60 min). The reaction is terminated by the addition of an acetonitrile containing internal standard. After centrifugation, aliquots of the samples are analyzed using LC-MS/MS to remove precipitated proteins (77, 82, 84, 85).

IN VITRO-IN VIVO EXTRAPOLATION

Prediction of human pharmacokinetic indices using *in vitro* data still remains a big challenge in the drug discovery and development process (86, 87). Rergarding the drug metabolic stability testing, IVIVE is an important tool for estimating the hepatic metabolic clearance of NCEs.

When analyzing metabolic stability data coming from different *in vitro* models, several important issues should be taken into consideration. First of all, both the rate of metabolism and metabolic enzymes activities observed *in vitro* should be close to *in vivo* conditions. Secondly, liver should be the major organ relating to NCE clearance and non-specific protein binding should be eliminated. Moreover, the compound concentration in the incubation mixture should be below the Michaelis-Menten constant (K_M) (2, 14).

Microsomal and hepatocyte stability assays are the key elements in high-throughput screening (HTS) of NCEs enabling the prediction of *in vivo* hepatic clearance. IVIVE procedure is based on the use of suitable scaling factors in order to transform the units of *in vitro* measured metabolic clearance to the rate of metabolism per gram of liver. Scaling factors depend on the *in vitro* system used. To obtain intrinsic clearance of the whole organ, the results are further multiplied by liver mass (Table I). There are appropriate scaling factors for data obtained with particular *in vitro* models, such as milligram of microsomal protein per gram of liver (MPPGL) for liver microsomes, number of hepatocytes per gram of liver (hepatocellularity, HPGL) for hepatocytes, and milligram of homogenate protein per gram of liver (Hom PGGL) when liver homogenates are employed (19, 45, 81, 88–91).

Many researchers have noticed that factors can influence prediction accuracy (92). In case of human MPPGL and HPGL values, inter-individual variability exists (*e.g.*, in case of human MPPGL – 32 mg g^{-1} *vs.* the most commonly used 45 mg g^{-1}, in case of HPGL – 99 × 10^6 cells g^{-1} *vs.* the most commonly used 120 × 10^6 cells g^{-1}) (19, 61, 68). In their study, Zhang *et al.* (61) proved that the mean value of MPPGL in 128 human livers was 39.46 mg g^{-1} and that up to 19-fold individual variations existed. Therefore, scaling factors should be matched to the population used.

Another problematic point relating to IVIVE are compounds that are extensively bound to plasma proteins (50, 69, 92). Obach (28) compared *in vivo* hepatic clearance predicted from *in vitro* models and CL_H values measured *in vivo* and stated that in some cases, the clearance values predicted from the *in vitro* HLM model were substantially lower than those observed *in vivo*. It is noteworthy that such discrepancy was related to compounds with high protein binding.

According to some authors, *in vitro* metabolic stability assays conducted with liver microsomes represent a limited tool in predicting *in vivo* hepatic clearance in humans (93). On the other hand, the cryopreserved human hepatocytes model is regarded as a universal system suitable for such prediction (14, 53). It was observed that when metabolic liver microsomes assays were employed, a tendency to clearance over-prediction appeared. This happened particularly in the case of compounds with low passive membrane permeability (25).

According to Chiba *et al.* (14), comparisons between the predicted and observed metabolic clearance in human using different datasets have indicated that the *in vitro* metabolic clearance obtained from both human liver microsomes and cryopreserved human hepatocytes systematically under-predicted *in vivo* metabolic clearance approximately 9 and 3 to 6-fold, resp.

Some authors investigated the direct *in vitro-in vivo* relationship of NCEs biotransoformation. Li *et al.* (69) evaluated the metabolism and pharmacokinetics of 4-(3,4,5-trimethoxybenzoyl)-2-phenylthiazole (SMART-H) that inhibited the proliferation of a variety of cancer cells *in vitro* and demonstrated that predicted clearance based on *in vitro* data correlated well with *in vivo* clearance in three animal models used in the experiment. In another study, Mukkavilli *et al.* (63), who examined a compound denoted as DNDI-VL-2098, a potential oral agent for visceral leishmaniasis, showed a close relationship between *in vivo* clearance in animal models and *in vitro* microsomal intrinsic clearance.

In vitro *interspecies differences of NCE metabolic stability.* – During *in vitro* metabolic stability testing, systems based on different preclinical species are employed (*i.e.*, human,

mouse, rat, dog and monkey). As different model organisms are used in the experiments, interspecies variations in metabolic stability are typically observed (9, 30, 38, 94–98).

One of the most important advantages of using different *in vitro* animal models in NCE metabolic stability assessment is the direct interspecies comparison of metabolic turnover rates and metabolic pathways. This may aid the selection of an appropriate animal model for further *in vivo* studies and to identify suitable surrogate species to humans, ensuring both cost and time reduction in the future *in vivo* experiments (35).

Interestingly, it has been regarded natural that monkey metabolism is most similar to that of humans; cynomolgus monkeys have been widely used in pharmacokinetic or drug-safety studies for that reason (99).

With reference to some drugs in use, Singh *et al.* (9) examined *in vitro* $t_{1/2}$ and $CL_{int,micr}$ of imipramine in human, rat, mouse and dog microsomes and observed the highest $t_{1/2}$ values

Table III. In vitro *half-life* ($t_{1/2}$) *and intrinsic clearance* (Cl_{int}) *data on some NCEs obtained with liver microsomes from various preclinical species*

Compound	Species										
	Human		Mouse		Rat		Monkey		Dog		
	Mean		Mean		Mean		Mean		Mean		
	$t_{1/2}$ (min)	Cl_{int} (μL mg^{-1} min^{-1})	$t_{1/2}$ (min)	Cl_{int} (μL mg^{-1} min^{-1})	$t_{1/2}$ (min)	Cl_{int} (μL mg^{-1} min^{-1})	$t_{1/2}$ (min)	Cl_{int} (μL mg^{-1} min^{-1})	$t_{1/2}$ (min)	Cl_{int} (μL mg^{-1} min^{-1})	Reference
SB639	59	–	3	–	6	–	–	–	60	–	94
I-387	24	–	37	–	42	–	15	–	29	–	95
SMART-H	17	–	<< 5	–	31	–	–	–	19	–	69
KBP-7018	–	90	–	40	–	70	–	440	–	120	30
SCY-078	≥ 42	34	≥ 125	≤ 11	≥ 125	≤ 11	–	–	≥ 29	≤ 48	100
AG-024322	720	–	–	–	18	–	2220	–	60	–	101
Orteronel®	> 60	4	> 60	3	> 60	8	> 60	3	> 60	4	98
E6201	36	–	84	–	89	–	–	–	70	–	96
FTY720-C2	–	18	–	23	–	80	–	20	–	6	97
FTY720-Mitoxy	–	18	–	2	–	8	–	135	–	1	97
S002-333	–	27	–	–	–	133	–	137	–	60	102
S004-1032	–	25	–	–	–	147	–	70	–	50	102
S007-1558	–	36	–	–	–	562	–	318	–	70	102
AZ'0908	–	19	–	–	–	14	–	–	–	93	103
PF-02413873	30	29	–	–	< 2	> 500	–	–	25	36	82

(and thus low $CL_{int,micr}$) in human and dog microsomes (65 and 47 min, resp.), whereas the lowest parameters were those in mouse and rat microsomes (11 and 5 min, resp.).

Table III gives the *in vitro* half-life and intrinsic clearance data of some NCEs obtained with liver microsomes from various preclinical species.

Pelkonen *et al.* (7), who compared metabolic stability of 55 validation compounds [European Centre for the Validation of Alternative Methods (ECVAM)/Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)] using human and rat liver homogenates and microsomes, observed that as regards metabolic stability, most compounds demonstrated similar behaviour in human and rat preparations. On the other hand, Kumar *et al.* (96) examined *in vitro* microsomal stability of a novel mitogen-activated protein kinase/extracellular signal-regulated kinase, kinase-1 (MEK1) inhibitor denoted as E6201, which showed faster metabolic clearance with HLM and the highest metabolic stability with RLM.

In another study Ahn *et al.* (95) conducted *in vitro* biotransformation of a novel antimitotic agent, I-387, in HLM, MLM, RLM, DLM and MnLM. They demonstrated that the test compound was most stable in RLM with the longest $t_{1/2}$ and degraded rapidly in MnLM with the shortest $t_{1/2}$. In case of HLM, $t_{1/2}$ values were more similar to those of MLM and DLM than those of the other two species.

Similarly, the highest *in vitro* $t_{1/2}$ was observed in RLM by Li *et al.* (69), who examined the metabolism of some 4-substituted methoxybenzoyl-aryl-thiazoles with the lead compound denoted as SMART-H. In HLM and DLM, *in vitro* half-lives were comparable, whereas the lowest $t_{1/2}$ was observed in MLM. Comparable *in vitro* $t_{1/2}$ between HLM and DLM were also reported by Venkatesh *et al.* (94) for compound SB639, a novel histone deacetylase inhibitor. Also, Wring *et al.* (100) evaluated the metabolic stability of SCY-078, the first-in-class orally active antifungal glucan synthesis inhibitor, in murine models of disseminated candidiasis in microsomes coming from different species. The authors concluded that the investigated compound demonstrated low clearance with rodent microsomes and low to moderate clearance in dogs and humans. It was observed that SCY-078 was most stable in rodent microsomes and most widely metabolized in DLM. Interestingly, Zainuddin *et al.* (98), who investigated Orteronel®, a CYP17A1 enzyme inhibitor, demonstrated the highest $CL_{int,micr}$ in rat microsomes, whereas the lowest values of this parameter were noticed in mouse and monkey microsomes.

Zhong *et al.* (101) investigated *in vitro* the microsomal metabolism of compound AG-024322, a novel cyklin-dependent kinase (CDK) inhibitor, employing HLM, RLM, DLM and MnLM. According to their results, the test compound depleted most extensively in RLM, followed by DLM. Moreover, comparable incubations in HLM and MnLM yielded significantly less extensive parent drug depletion. On the other hand, when Huang *et al.* (30) investigated the *in vitro* microsomal stability of KBP-7018, a new tyrosine kinase inhibitor candidate for treatment of idiopathic pulmonary fibrosis, the lowest $CL_{int,micr}$ was recorded in MLM and RLM, whereas the highest values were observed for MnLM.

Enoru *et al.* (97) assessed the preclinical metabolism of new blood-brain-barrier penetrant fingolimod analogues: FTY720-C2 and FTY720-Mitoxy. The *in vitro* intrinsic clearance of FTY720-C2 was low in dog, moderate in mouse, monkey and human, and high in rat, whereas in the case of the second analogue, FTY720-Mitoxy, the intrinsic clearance was found to be low in mouse, rat and dog, moderate in human and high in monkey.

In another study, Saxena *et al.* (102) characterized the metabolism of S002-333 [2-(4′-methoxy-benzenesulfonyl)-2,3,4,9-tetrahydro-1*H*-pyrido(3,4-*b*)indole-3-carboxylic acid amide] and its enantiomers, S004-1032 and S007-1558, using liver microsomes from different species. In case of compound S002-333, the highest CL_{int} values were noted in MnLM and were comparable to the values for RLM. The lowest intrinsic clearance values were reported for HLM. In the case of enantiomers, the highest CL_{int} was observed for RLM, whereas the lowest were those for rabbit liver microsomes.

Bylund and Bueters (103) evaluated compound AZ'0908, a novel microsomal prostaglandin E synthase-1 inhibitor intended for oral administration. The authors found that Cl_{int} values in liver microsomes were significantly higher in dog than in rat and human. It is noteworthy that Bungay *et al.* (82), who investigated *in vitro* stability of PF-02413873 (4-[3-cyclopropyl-1-(methylsulfonylmethyl)-5-methyl-1*H*-pyrazol-4-yl]oxy-2,6-dimethylbenzonitrile), a non-steroidal progesterone receptor antagonist, observed the highest Cl_{int} values in RLM, whereas in DLM and HLM clearance values were significantly lower than in rat.

In summary, the above data show clearly that compounds exhibit interspecies variability in their metabolic stability, since their *in vitro* half-lives and intrinsic clearance values usually vary greatly when different human or animal microsomes are used. It is therefore difficult to make compound metabolic stability cross-species predictions and each model should be tested to obtain reliable data, especially in the context of further *in vivo* experiments. These differences in drug metabolism result from many factors, such as differences in the primary sequence and levels of CYP isoforms between various species as well as differences in their hepatic metabolism (15, 104, 105).

CONCLUSIONS

During the drug discovery and development process, preclinical metabolic stability studies are essential to determine pharmacological and toxicological profiles of new chemical compounds. In the early phases of drug discovery, NCEs metabolic properties are determined using different *in vitro* systems. The obtained *in vitro* metabolism data are further used to predict the *in vivo* clearance of compounds. Moreover, preliminary *in vitro* experiments are useful in the selection of appropriate animal models for further *in vivo* studies and to identify suitable surrogate species to humans, ensuring both cost and time reduction in the future experiments. Despite the fact that *in vitro-in vivo* extrapolation procedures raise some questions, scientists are still working on improving this strategy to achieve more satisfactory predictions.

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