OULU 2015
D 1328

ACTA UNIVERSITATIS OULUENSIS


*Jouko Uusitalo*

# THE ROLE OF DRUG METABOLISM IN DRUG DISCOVERY AND DEVELOPMENT – CASE OSPEMIFENE

*UNIVERSITY OF OULU GRADUATE SCHOOL;*
*UNIVERSITY OF OULU,*
*FACULTY OF MEDICINE;*
*MEDICAL RESEARCH CENTER OULU;*
*OULU UNIVERSITY HOSPITAL*

ACTA UNIVERSITATIS OULUENSIS
D Medica 1328

*JOUKO UUSITALO*

# THE ROLE OF DRUG METABOLISM IN DRUG DISCOVERY AND DEVELOPMENT – CASE OSPEMIFENE

Academic dissertation to be presented with the assent of the Doctoral Training Committee of Health and Biosciences of the University of Oulu for public defence in Auditorium F202 of the Faculty of Medicine (Aapistie 5 B), on 4 December 2015, at 12 noon

UNIVERSITY OF OULU, OULU 2015

Acta Univ. Oul. D 1328, 2015

Supervised by
Docent Miia Turpeinen
Professor Olavi Pelkonen

Reviewed by
Professor Markku Koulu
Docent Mikko Koskinen

ISBN 978-952-62-1021-6 (Paperback)
ISBN 978-952-62-1022-3 (PDF)

ISSN 0355-3221 (Printed)
ISSN 1796-2234 (Online)

Cover Design
Raimo Ahonen

JUVENES PRINT
TAMPERE 2015

**Uusitalo, Jouko, The role of drug metabolism in drug discovery and development – Case Ospemifene.**

University of Oulu Graduate School; University of Oulu, Faculty of Medicine; Medical Research Center Oulu; Oulu University Hospital

*Acta Univ. Oul. D 1328, 2015*

University of Oulu, P.O. Box 8000, FI-90014 University of Oulu, Finland

***Abstract***

Drug metabolism is one of the most important events a drug faces after administration. Traditionally, drug metabolism has only been considered as a major clearance and elimination step in the pharmacokinetics of a drug. However, drug metabolism is also one of the important factors behind safety and toxicity issues in drug discovery and development. Some of the mechanisms behind metabolism-related toxicity we do understand well while others, especially the role of reactive metabolites, need further research. The thesis reviews the role of drug metabolism in the drug discovery and development process from the point of view of metabolism and metabolites. Special emphasis is put on reviewing the metabolism behind human toxicity and safety, and the roles of circulating and reactive metabolites in particular.

Ospemifene is a nonsteroidal selective estrogen receptor modulator recently approved for the treatment of vulvar and vaginal atrophy in postmenopausal women with moderate to severe dyspareunia. The present study characterized the *in vitro* and *in vivo* metabolism and potential drug interactions of ospemifene. The principal human metabolites were identified and the adequacy nonclinical animal exposure was evaluated. The major human cytochrome P450 enzymes involved in the formation of principal metabolites were also identified and the clinical consequences assessed. Finally, the interaction potential of ospemifene as a cytochrome P450 enzyme inducer or inhibitor was investigated. As a result, ospemifene was considered to be safe drug from a metabolic interaction point of view.

This study was part of the drug development program of ospemifene and practically all of the *in vitro* study data were included in the marketing authorization application of ospemifene. Ospemifene was also a case molecule in the development of new methodologies to study drug metabolism and drug-drug interactions.

*Keywords:* ADME, bioanalysis, Cytochrome P450 enzyme system, DMPK, drug metabolism, drug safety, drug-drug interactions, ospemifene, pharmaceutical analysis, pharmacokinetics, reactive metabolites

**Uusitalo, Jouko, Lääkeainemetabolian merkitys lääkekehityksessä – esimerkkitapauksena ospemifeeni.**
Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta; Medical Research Center Oulu; Oulun yliopistollinen sairaala
*Acta Univ. Oul. D 1328, 2015*
Oulun yliopisto, PL 8000, 90014 Oulun yliopisto

## *Tiivistelmä*

Lääkeainemetabolia on lääkeaineen farmakokinetiikassa tärkeä puhdistuma- ja eliminaatioaskel, jonka rooli on ymmärretty varsin hyvin. Lääkeainemetabolialla on myös merkittävä vaikutus lääkeaineen toksisuuteen ja lääkkeen käytön turvallisuuteen. Osa lääkeainemetaboliaan liittyvistä toksisuusmekanismeista selvitetty hyvin, mutta erityisesti reaktiivisiin metaboliitteihin liittyvä osa vaatii vielä tutkimusta. Tämän työn kirjallisuusosassa katselmoidaan lääkeainemetabolian merkitystä lääkekehitysprosessissa painottaen erityisesti lääkeainemetabolian sekä reaktiivisten ja verenkierrossa kiertävien metaboliatuoteiden vaikutusta toksisuuteen ihmisellä ja merkitystä turvalliseen lääkkeiden käyttöön.

Ospemifeeni on uusi ei-steroidinen selektiivinen estrogeenireseptorimodulaattori, joka on hyväksytty yhdynnänaikaisesta kivusta kärsivien postmenopausaalisten naisten vulvan ja vaginan limakalvojen kuivumisen hoitoon. Tässä tutkimuksessa selvitettiin ospemifeenin lääkeainemetaboliaa ihmisellä ja koe-eläimillä sekä mahdollisia lääkeinteraktioita. Tutkimuksessa tunnistettiin tärkeimmät metaboliitit ihmisellä ja arvioitiin eläinkokeissa käytettyjen koe-eläinten altistumisen kattavuus niille. Työssä selvitettiin myös tärkeimmät päämetaboliitteja katalysoivat *sytokromi* P450 -entsyymit ja arvioitiin löydösten kliinistä merkitystä. Lisäksi tutkittiin aiheuttaako ospemifeeni lääkeinteraktioita muille lääkeaineille indusoimalla tai inhiboimalla *sytokromi* P450 -entsyymejä. Tutkimustulosten perusteella ospemifeenia voidaan pitää lääkeainemetabolian suhteen turvallisena lääkkeenä.

Tämä tutkimus oli osa ospemifeenin lääkekehitysohjelmaa ja käytännössä kaikki tutkimustyön *in vitro* -tietoaineisto oli mukana ospemifeenin myyntilupa-hakemuksissa lääketurvallisuusviranomaisille. Ospemifeenia käytettiin tutkimustyön aikana myös yhtenä esimerkkimolekyylinä kehitettäessä uusia menetelmiä lääkeainemetabolian ja lääkeinteraktioiden tutkimiseen.

*Asiasanat:* ADME, bionalytiikka, DMPK, farmakokinetiikka, lääkeaineanalytiikka, lääkeainemetabolia, lääkeinteraktiot, lääketurvallisuus, ospemifeeni, reaktiiviset metaboliitit, sytokromi P450 -entsyymijärjestelmä

*To my family*

# Acknowledgements

This thesis was carried out at the University of Oulu, Faculty of Medicine, Department of Pharmacology and Toxicology and Faculty of Science, Department of Chemistry during the years 1998–2015.

I am thankful to Prof. Emeritus Olavi Pelkonen for the scientific supervision of the experimental work and enthusiastic guidance when commercializing it. It has always been a real pleasure to work with Olavi and feel his warmness and advocacy. I am equally thankful to my principal thesis supervisor Doc. Miia Turpeinen. It is certain that without her activity the writing of this thesis would have never been even started. I thank Miia for her eternal kindness, encouragement and patience during the long writing process. I am also thankful to my former supervisors from Department of Chemistry, Doc. Jorma Jalonen and Prof. Emeritus Erkki Rahkamaa, for their support and wisdom.

I thank Prof. Markku Koulu and Doc. Mikko Koskinen for their prompt pre-examination and invaluable comments. I am also thankful to Anna Vuolteenaho for the proficient revision of English language. I wish to thank all the co-authors of the original publications for their precious contribution to this work. I want to especially acknowledge the important role of Doc. Ari Tolonen. I am grateful to my former colleaques at the Laboratory of Mass Spectrometry of University of Oulu and at Novamass Ltd. for their skillful co-operation and joyous atmosphere to work with.

I am warmly thanking my family and friends for their endorsement and love. I am grateful to my employer Technopolis Plc for kindly admitting me to finish the thesis and my colleaques at work for their continuous cheer.

The collaboration of Hormos Medical Corp. and QuatRx Pharmaceuticals Company are highly appreciated. This study was funded by Tekes – the Finnish Funding Agency for Innovation, Kauhajoki Cultural Foundation, Tauno Tönning Foundation and Emil Aaltonen Foundation. I am grateful for their valuable support.

Oulu, October 2015 Jouko Uusitalo

# Abbreviations

| | |
|---|---|
| Acyl-CoA | Acyl-coenzyme A |
| ADMET | drug absorption, distribution, metabolism, excretion and toxicity |
| ADR | adverse drug reaction |
| AKR | aldo-keto reductase |
| AME/MB | absorption, metabolism and excretion |
| AMS | accelerator mass spectrometry |
| AO | aldehyde oxidase |
| AOR | aldehyde oxidoreductase |
| AUC | area under concentration-time curve |
| AZ | AstraZeneca |
| BCRP | breast cancer resistance protein |
| BSEP | bile salt expoirt pump |
| CAR | constitutive androstane receptor |
| CES | carboxylesterase |
| cLogD | calculated distribution coefficient |
| Cmax | maximum concentration |
| CVB | covalent binging |
| CYP | cytochrome P450 enzyme |
| DDI | drug-drug interactions |
| DILI | drug-induced liver injury |
| DMPK | drug metabolism and pharmacokinetics |
| EM | exposure multiples |
| EMA | European Medicines Agency |
| FDA | US Food and Drug Administration |
| FIH | first-in-human |
| $f_m$ | fraction of metabolite formed from the absorbed parent |
| FMO | flavin-containing mono-oxygenase |
| FTMS | Fourier transform mass spectrometry |
| GLP | good laboratory practice |
| GSH | glutathione |
| GSK | GlaxoSmithKline |
| HPLC | high resolution liquid chromatography |
| HTS | high-throughput screening |
| IC50 | half maximal inhibitory concentration |
| ICH | International conference of harmonization |

| | |
|---|---|
| IDR | idiosyncratic drug reaction |
| IMS-MS | ion mobility spectrometry combined with mass spectrometry |
| IND | investigational new drug |
| IVIVE | in vitro - in vivo extrapolation |
| KCN | potassium syanide |
| Ki | inhibition constant |
| Km | Michaelis constant |
| LC/MS | liquid chromatography combined with mass spectrometry |
| LC/RAD | liquid chromatography combined with radioactivity detection |
| LC/UV | liquid chromatography combined with ultraviolet detection |
| logP | partition coefficient |
| LSC | liquid scintillator counting |
| M&A | mergers and acquisitions |
| M/P | metabolite to parent ratio |
| MAO | monoamine oxidase |
| MB | mass balance |
| MBI | mechanism-based inhibition |
| MDI | metabolism-dependent inhibition |
| MDR | multiple drug resistance protein |
| MIST | metabolites in safety testing |
| MRP | multidrug resistance associated protein |
| MS/MS | tandem mass spectrometry |
| $MS^E$ | high and low collision energy every second scan mass spectrometry |
| $MS^n$ | multiple step tandem mass spectrometry |
| MTD | maximum tolerated dose |
| NADPH | reduced nicotinamide adenine dinucleotide phosphate |
| NCE | new chemical entity |
| NDA | new drug application |
| NME | new molecular entity |
| NMR | nuclear magnetic resonance |
| NOAEL | no observable adverse events level |
| OAT | organic anion transporter |
| OATP | organic anion transporting peptides |
| OCT | organic cation transporter |
| OXR | oxidoreductase |
| PAPS | 3'-Phosphoadenosine-5'-phosphosulfate |
| PBPK | physiologically based pharmakokinetic |

| | |
|---|---|
| P-gp | P-glycoprotein |
| PK | pharmacokinetics |
| pKa | acid dissociation constant |
| PPB | plasma protein binding |
| PSA | polar surface area |
| PXR | pregnane X receptor |
| Q&A | questions and answers |
| QTOF | quadrupole time of flight |
| QWBA | quantitative whole body autoradiography |
| R&D | research and development |
| RAD | radioactivity detection |
| S/N | signal to noise ratio |
| S9 | cellular fraction containing cytosol and microsomes. |
| SERM | selective estrogen receptor modulator |
| TDI | time-dependent inhibition |
| thioTEPA | N,N'N'-triethylenethiophosphoramide |
| TOFMS | time of flight mass spectrometry |
| UDPGA | UDP-glucuronic acid |
| UGT | UDP-glucuronosyltransferases |
| UHPLC | ultra high resolution liquid chromatography |
| UPLC | ultra performance liquid chromatography |
| WBA | whole body autoradiography |
| XOR | xanthine oxidoreductase |

# List of original publications

This thesis is based on the following publications, which are referred throughout the text by their Roman numerals:

I Makowiecki J, Tolonen A, Uusitalo J & Jalonen J (2001) Cone voltage and collision cell collision-induced dissociation study of triphenylethylenes of pharmaceutical interest. Rapid Commun Mass Spectrom 15(17): 1506–1513.

II Tolonen A, Koskimies P, Turpeinen M, Uusitalo J, Lammintausta R & Pelkonen O (2013) Ospemifene metabolism in humans *in vitro* and *in vivo*: metabolite identification, quantitation, and CYP assignment of major hydroxylations. Drug Metabol Drug Interact 28(3): 153–161.

III Turpeinen M, Uusitalo J, Lehtinen T, Kailajarvi M, Pelkonen O, Vuorinen J, Tapanainen P, Stjernschantz C, Lammintausta R & Scheinin M (2013) Effects of ospemifene on drug metabolism mediated by cytochrome P450 enzymes in humans *in vitro* and *in vivo*. Int J Mol Sci 14(7): 14064–14075.

IV Lehtinen T, Tolonen A, Turpeinen M, Uusitalo J, Vuorinen J, Lammintausta R, Pelkonen O & Scheinin M (2013) Effects of cytochrome P450 inhibitors and inducers on the metabolism and pharmacokinetics of ospemifene. Biopharm Drug Dispos 34(7): 387–395.

V Uusitalo J, Turpeinen M, Tolonen A, Koskimies P, Lammintausta R & Pelkonen O (2015) Metabolism and metabolite profiles *in vitro* and *in vivo* of ospemifene in humans and preclinical species. Drug Metabol Pers Ther 2016. DOI: 10.1515/dmdi-2015-0020. [Epub ahead of print.]

# Table of contents

# 1 Introduction

Drug discovery and development is a 10–15 year and multi-billion dollar process that leads to the acceptance of about 30 new drugs in the market each year. The process is currently in a discontinuity state as the earlier high-yielding blockbuster model is no longer fully valid and the new model with narrower therapeutic segments is finding its form. At the same time, patents of the ex-blockbusters are expiring, regulatory requirements increasing and the cost of the process rising. All easy targets seem to already have efficient drugs and the industry is desperately seeking productivity in finding new druggable targets and unique chemistry. Despite the huge efforts, most of the drug development programs fail, and the main reasons are related to efficiency and safety.

Drug metabolism is one of the most important events a drug faces after administration. Traditionally, drug metabolism has only been considered as a major clearance and elimination step in the pharmacokinetics of a drug. At the moment we understand that role remarkably well, but that is not all. We have now learned that drug metabolism is also one of the important factors affecting safety and toxicity issues currently encountered in drug discovery and development. Some of the mechanisms behind metabolism-related toxicity we do understand well while others, especially the role of reactive metabolites, need further research.

The literature review examines the role of drug metabolism in the drug discovery and development process from the point of view of metabolism and metabolites, not from the traditional point of view of a drug and its pharmacokinetics. Special emphasis is put on reviewing the metabolism factors behind human toxicity and safety, and the roles of circulating and reactive metabolites in particular.

The present study aimed to characterize the *in vitro* and *in vivo* metabolism and potential drug interactions of a newly accepted drug, ospemifene. A further aim was to evaluate the clinical safety of ospemifene considering the found metabolism characteristics and drug interaction potential. The study was part of the drug development program of ospemifene and most of the study results were included in the marketing authorization application dossier delivered to regulatory authorities. Additionally, the study molecule was one of the case molecules used in the development of new biochemical and analytical methodologies to study drug metabolism and related drug interactions.

# 2 Review of the literature

## 2.1 Drug discovery and development

The US Food and Drug Administration (FDA) approved 27 new medicines (25 small molecules and 2 biologics) in 2013, (FDA 2014, Mullard 2014a). In 2014, the FDA approved 41 new medicines including 11 biologics, which is the highest number of approvals since 1997 (FDA 2015, Mullard 2015). Since 2000 the average number of new approvals has been 27.3 new molecular entities (NMEs, covering both small molecule drugs and biologics) per year. The trend seems to be upwards from the all-time-low in 2007, as can be seen from Fig. 1. The European Medicines Agency (EMA) approval history also shows positive trend as 41 medicines containing new active substances were approved in 2014 compared to 38 in 2013, 35 in 2012, 25 in 2011 and 15 in 2010 (EMA 2014, EMA 2015a). One of the small molecules approved by FDA in 2013 and by EMA in 2014 was the study case molecule ospemifene (FDA 2014, EMA 2015b).

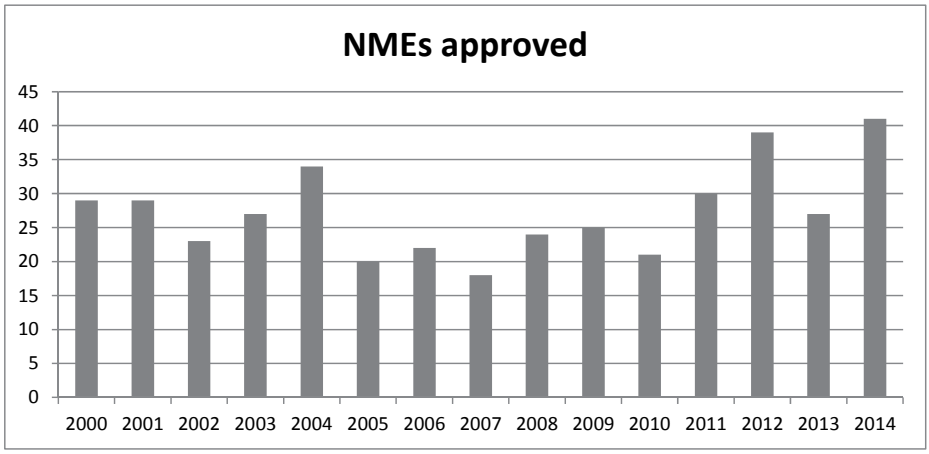


**Fig. 1. New molecular entities approved by FDA since 2000 (FDA 2014 & 2015).**

Historically, the pharmaceutical industry has produced a total of 1,494 NMEs to the market since the year 1827 (Kinch *et al.* 2014, FDA 2015). After 1950 the number is 1,406 new drugs to the market (Munos 2009, FDA 2015). Globally, there have been 261 pharmaceutical companies that have produced these new medicines (having at least one registration by Munos 2009), and 105 of them still exist after mergers and acquisitions (total 137 M&A), and 19 liquidations (Munos

2009). Today, two thirds of all NMEs are controlled by the top 10 pharmaceutical companies (Kinch *et al.* 2014).

The small molecule drug discovery and development process from hit identification to product launch takes about 13.5 years and costs approximately USD900 million as out-of-pocket expenses (Paul *et al.* 2010). The most expensive phase is clinical Phase III costing USD235 million, on average. However, taking into account the cost of capital, the high project discontinuation rate and the length of the process cycle to produce one new approved drug, each new product launch had cost USD1.8 billion. When the cost of capital etc. is considered, the most expensive phase is actually lead optimization, costing in excess of USD400 million (Paul *et al.* 2010). And this is not all; additional costs are incurred from e.g. exploratory discovery research, post-approval R&D and overheads to supporting functions. In 2008, the total cost for new product launch was calculated to be around USD3.9 billion (Munos 2009).

To compensate for the high costs there must be high returns. It has been forecasted that global drug sales will exceed USD1 trillion in 2014 (Mullard 2014b). Growing sales are not the full picture though. At the same time the patents of many blockbuster drugs are expiring, giving rise to fierce generic competition and up to 90% revenue erosion for the ex-blockbuster. In 2012, an estimated drop of USD33 billion in revenue hit previous blockbusters including the biggest ever selling drug, Pfizer's Lipitor (Tse & Kirkpatrick 2013). Naturally, the loss of revenue impacts the industry: over 30,000 employees were laid off in 2012 and 2013.

The fact that blockbusters of the past are generic mainstream medications of today creates extra hurdles for the industry in terms of discovery and development of new medications. It forces the discovery process into new unexplored areas where success is not granted. It affects adoption and reimbursement of newly approved medications as old ones are still very good (and cheap). It makes the approval process tight and regulators more cautious, demanding new medications that have more benefits and are less risky than existing (generic) ones (Scannell *et al.* 2012). As the blockbuster model has become less viable, the pharmaceutical industry is forced to search drugs for niche patient groups and rare diseases (Kakkar & Dahiya 2014).

### 2.1.1 Drug discovery and development process

The small molecule drug discovery and development process consists of three major steps: discovery research, preclinical testing and clinical trials. While clinical trials were seldom conducted with more than one candidate drug in the past, nowadays it is not rare to start with couple of clinical candidates. Preclinical testing, however, frequently involves several or even tens of potential drug candidates called lead molecules or plain leads. In the discovery phase, hundreds hit molecules or even thousands of screened molecules are filtered down to a number promising leads or lead families for preclinical testing. Typically, pharmaceutical companies have tens and drug developing biotech companies several drug development programs going on in different phases and parallel activities within a program for clinical drug candidates or preclinical leads, back-up compounds and major metabolites. In the discovery phase, tens of molecules can be simultaneously under planning, synthesis, structure optimization, physicochemical characterization and *in vitro* testing for efficacy or ADMET (drug absorption, distribution, metabolism, excretion and toxicity) properties (Pritchard *et al.* 2003, Kramer *et al.* 2007, Ballard *et al.* 2012).

The drug discovery phase starts with target biology activities. A new or existing target is identified and the approach is validated. One desired outcome is simple and robust *in vitro* assay (e.g. receptor binding or activity inhibition) that can be utilized to test and rank a large number of different chemicals in high-throughput format. This screening of molecular libraries (e.g. natural product or combinatorial chemistry library) against target *in vitro* assay is called hit identification. The purpose of hit identification is to find several different classes of molecules that give positive response (hit) from the test (Pritchard *et al.* 2003, Kramer at al 2007, Ballard *et al.* 2012). Confirmed hit molecules having adequate positive metrics (e.g. binding affinity or $IC_{50}$) from the assay are then subjected to hit expansion towards early lead molecules. Several hit compound series are synthesized and explored using target *in vitro* assays (efficacy and selectivity tests) to define structure-activity relationship. Key physicochemical and ADMET properties are also measured or computed (van de Waterbeemd & Gifford 2003, Kirchmair *et al.* 2015). The lead-likeness of the best hit molecules includes the following criteria (Pritchard *et al.* 2003, Kramer at al 2007, Ballard *et al.* 2012):

- Very high affinity or other potency metrics.
- Smaller molecular weight and lower lipophilicity compared to general drug-like rules (room for chemical optimization).
- Reasonable predicted pharmacokinetics (PK).
- Patentable structure without potentially toxic substructures, i.e. toxicophores.

During the lead optimization phase the structures of selected leads are chemically tuned by medicinal chemists to produce optimal combination of efficacy, selectivity and pharmacokinetics without suspicion of toxicity. Mostly *in vitro* testing is utilized at this point, but some *in vivo* animal testing is also conducted for the most promising leads (Pritchard *et al.* 2003, Kramer at al 2007, Ballard *et al.* 2012). Typically, the selected lead compounds for preclinical development have 42 Da higher molecular weight and 0.4 logP-unit higher lipophilicity than the compounds entering lead optimization had (Morphy 2006).

The aim of the preclinical phase is to come down to one clinical candidate having such an animal safety profile that administration to human is without risks. Most of the studies are *in vivo* pharmacological and toxicological animal studies, and *in vitro* safety studies under good laboratory practice (GLP) demanded by the regulator. Drug metabolism and pharmacokinetics (DMPK) is studied with radiolabeled study compound to define mass balance and exposure to circulating metabolites (Pritchard *et al.* 2003, Kramer at al 2007, Ballard *et al.* 2012).

Clinical development has three phases: I, II and III. The first one with healthy volunteers should prove the safety and tolerability of the candidate drug. It also gives confirmation on the bioavailability and pharmacokinetics of the compound. The second phase is conducted with patient group(s) to create clinical proof of efficacy. The last phase produces definitive clinical proof of principle in large patient group(s) with long treatment duration. It also indicates whether the long-term administration has safety or tolerability issues. Additional clinical studies can be conducted with patients or volunteers to examine drug-drug interactions. After marketing authorization, the safety surveillance (pharmacovigilance) of accepted drug continues and this is commonly called clinical Phase IV trial.

### *2.1.2 Attrition in drug development*

Drug development suffers from high attrition (discontinuation of clinical development). In recent years, the attrition has increased as the productivity in drug development phases has dropped dramatically from overall 10% of

compounds passing all the clinical development phases in the 1990s to just 6% in 2009–2010 (Kola & Landis 2004, Khanna 2012). This means that for every 100 investigational new drugs (IND) entering clinical Phase I trials only six make it to the market today. The attrition rate comparison in Fig. 2 clearly shows that attrition has been coming down from 40% to 30% in Phase I, but for all the other phases the trend has been upwards, and fewer compounds enter the next phase. Very alarming is that in 2010, even 75% of compounds entering Phase II clinical trials and over 50% of compounds entering Phase III are discontinued. In the regulatory review stage after new drug application (NDA), almost 30% of compounds are still rejected.

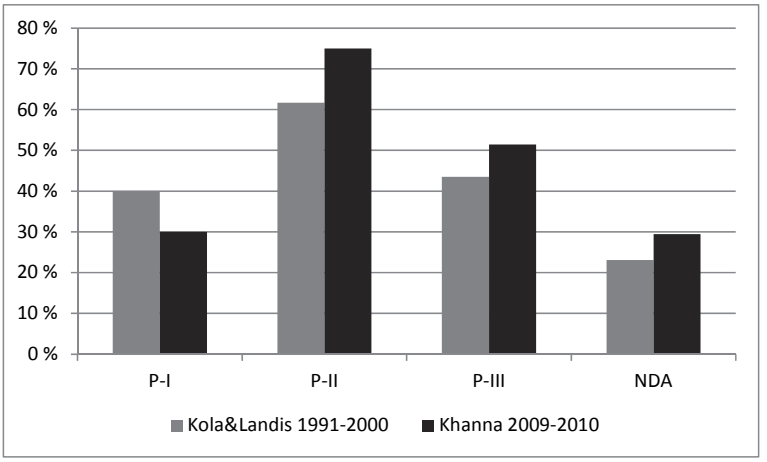


**Fig. 2. Attrition rate by clinical phase (data from Kola & Landis 2004, Khanna 2012).**

However, this negative image is not the full consensus of attrition rates in the pharmaceutical industry. The development pipelines of the 50 largest pharmaceutical companies (by sales) were analyzed in 2009 and it was concluded that the overall success rate from IND to market approval in US was 19% (DiMasi *et al.* 2010). The investigation included 1,225 compounds entering first clinical testing during 1993–2004 and the approval/failure was tracked until June 2009. The most recent study examines 835 drug developers including biotech companies, specialty pharmaceutical companies and large pharma from 2003 to 2011, showing the success rate to be 10.4% (Hay *et al.* 2014).

What are the reasons behind attrition in clinical development and have they changed over the time? For years we have seen the picture in Figure 3 showing how pharmacokinetics was for decades believed to be the main reason for attrition

(Kola & Landis 2004, Prentis *et al.* 1988). However, the alarming information of Kola and Landis is only based on two years of program cancellations (1991 and 2000) and the analysis of Prentis includes 77 anti-infectives rejected due to unexpectedly poor human PK. The situation changes dramatically if these compounds are removed, as pointed out by Kennedy (1997) and Kubinyi (2003). Figure 4 shows the data of 1964–1985 (Prentis *et al.* 1988) now excluding anti-infectives and the data of 1991 and 2000 (Kola & Landis 2004) has been averaged. Additionally, recent reports about Phase II and Phase III failures have been added together and included in the figure (Arrowsmith 2011a, Arrowsmith 2011b, Arrowsmith & Miller 2013). In Figure 3 and 4, the 'safety' column includes both animal toxicity reasons and human adverse events, and the 'commercial' column both commercial and financial reasons. The PK column also contains formulation reasons for the year 2000.

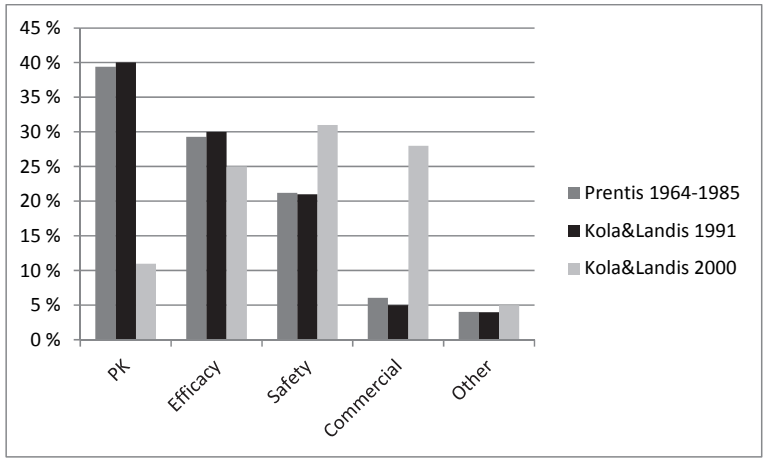


**Fig. 3. Traditional view for reasons of drug development attrition in 1964–2000 (data from Prentis *et al.* 1988, Kola & Landis 2004).**

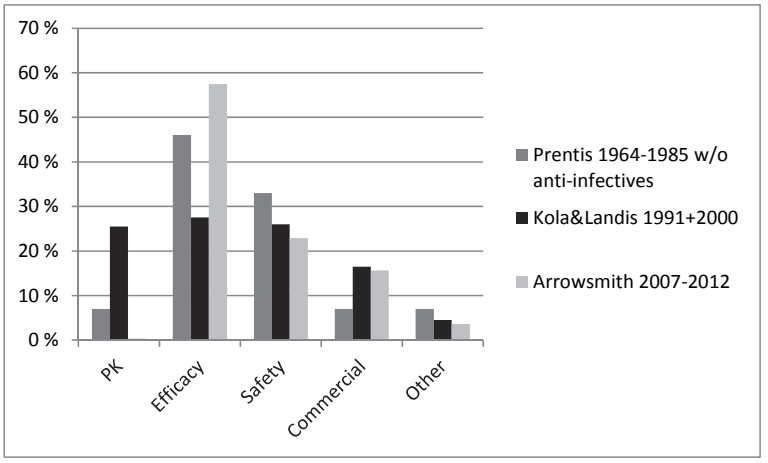


**Fig. 4. Reasons for drug development attrition in 1964–2012 (data from Prentiss *et al.* 1988, Kola & Landis 2004, Arrowsmith 2011a, Arrowsmith 2011b, Arrowsmith & Miller 2013).**

The main failure issue has actually been, and still is, efficacy or rather lack of it. The industry can very well design drug candidates that behave nicely within the body causing less and less harm, but unfortunately they also seem to cause less and less benefit. Interestingly, in the 1990s, pharmacokinetics seemed to cause much trouble and at the same time, lack of efficacy was relatively low. Concerning the causes for lack of efficacy, a detailed analysis of 44 Phase II programs was performed by Pfizer, with the conclusion that many programs failing due to lack of efficacy had no clear reasons why. They could not state if the Phase II failure was caused by the drug not producing pharmacologically relevant activity in the target (e.g. failure to reach the target or bind to the target) or if the target itself was relevant or not for the indication (Morgan & van der Graaf 2012).

It can be seen from Fig. 4 that the industry was capable of solving problem of poor human PK. Pharmacokinetics as reason for attrition does not exist anymore due to huge efforts to understand drug absorption, metabolism, distribution and excretion during the last few decades. The problems with safety have also decreased by about one third over the decades, probably due to increased understanding about toxicity mechanisms and the reasons behind adverse events. However, this issue is far from solved: 35% of failures in Phase III during 2011–12 were due to safety issues (Arrowsmith & Miller 2013). The data analysis in 1999 from 12 pharmaceutical companies of 79 discontinued clinical drug

candidates due to safety reasons indicates the organ systems mainly involved in safety-related failures: Cardiovascular, liver and nervous system 22% each, immunity and renal both about 10%, and the rest 14% (Olson *et al.* 2000, Watkins 2011). A recent study by AstraZeneca shows quite similar results for their safety attrition during 2005–2010 with the notable exception that hepatotoxicity accounts only for about 13% of the safety failure reasons (Cook *et al.* 2014). The same study also reveals safety being the most important project closure reason in preclinical and Phase I clinical development (82% and 62% of the closures, respectively), whereas efficacy, not surprisingly, dominates in the Phase II (proof of concenpt) closures: 57% in Phase IIa and 88% in Phase IIb (Cook *et al.* 2014).

### *2.1.3 The productivity gap*

One could easily conclude that productivity issues of the pharmaceutical industry lie in clinical drug development due to high attrition rates. This is not the case, however. The problem is actually the discovery phase which is failing to produce safe and efficient preclinical leads and drug candidates (Scannell *et al.* 2012, Dimitri 2011). Over the last decades there have been huge apparent improvements in efficiency, quality and throughput in many discovery areas, like high-throughput screening (HTS) methods, combinatorial synthesis, computerized tools and ADMET characterization. This should have increased the probability of success in the clinical phase, which has not happened (DiMasi *et al.* 2010), but the overall R&D efficiency has actually declined (Munos 2010).

There are opinions that the industry industrialized the wrong set of discovery activities with HTS methodologies, which has led to screening of similar sets of compounds throughout the industry (Scannell *et al.* 2012). This has two dramatic consequences. First, unique patentable chemistry is difficult to obtain and secondly, with limited chemical starting space the screening hits minimal local optima rather than finding maximal global optimum. In fact, HTS-based screening for new targets is two times more likely to fail than screening for known targets (Keserü & Makara 2009). The current approach values *in vitro* potency and selectivity the most, but it has been shown that *in vitro* potency has negative correlation with good ADMET properties and additionally, may have negative correlation with *in vivo* efficiency (Gleeson *et al.* 2011). One approach to overcome the productivity gap is the Chorus model of Eli Lilly and company (Owens *et al.* 2015). The Chorus model operates in biotech-like networked manner inside Big Pharma and aims for fast *in vivo* proof-of-concept. The model

has demonstrated clear improvement in productivity compared to the traditional approach in terms of substantial savings in time to market and money spent with increased probability to market launch (Owens *et al.* 2015). On the other hand, Big Pharma could improve their overall productivity by a remarkable 30% if early killing of poor assets were improved by only 15% (Smietana *et al.* 2015).

## 2.2 Drug metabolism

The pharmaceutical industry has invested massively in discovery activities over the last couple of decades. One of the investment areas has been early screening and optimization of drug metabolism and pharmacokinetics (DMPK); as a consequence, poor human pharmacokinetics has vanished from the list of reasons for clinical attrition, as shown in Figure 4. Good pharmacokinetics is not only an issue of metabolism; it also involves multivariate optimization of physicochemical properties and structural features that affect solubility and dissolution, permeability and absorption, and metabolism and related interactions (Ballard *et al.* 2012, Bergstöm *et al.* 2013, Yusof *et al.* 2014).

### *2.2.1 Importance of lipophilicity*

General relationships between physicochemical properties and pharmacokinetics are well understood (Obach *et al.* 2008a) and thanks to pioneering proposals of Lipinski *et al.* (1997) there are now numerous sets of rules-of-thumb on how to optimize physicochemical properties and what kind of cut-offs should be applied for different properties (Lipinski *et al.* 1997, Gleeson 2008, Johnson *et al.* 2009, Choy & Prausnitz 2011). These rules of thumb are usually combined to the standard set of cheminformatics tools of medicinal chemistry (Muchmore 2010, Cumming *et al.* 2013). An experienced medicinal chemist can interpret the screening outputs of modified compounds to a remarkable extent. For example, adding compound lipophilicity tends to increase permeability, but decrease solubility, increase the rate of metabolism and the number of metabolites, and raise plasma protein binding (Gleeson 2008, Varma *et al.* 2010, Meanwell 2011). On the other hand, adding hydrophilicity and ionizable groups to compounds – like drug metabolism does – may lead to poor absorption and tissue distribution due to decrease in membrane penetrability, transporter-mediated kinetics and rapid renal clearance (Varma *et al.* 2009, Obach 2013).

High lipophilicity is also associated with compound toxicity (Leeson & Springthorpe 2007, Hughes *et al.* 2008, Muchmore *et al.* 2010). In a joint study of four Big Pharma companies (AZ, Eli Lilly, GSK and Pfizer) concerning 812 oral compounds nominated for development between 2000 and 2010, it was found out that compounds reaching Phase II had an average calculated LogP of 3.1, whereas for compounds failing due to clinical safety concerns the average value was 3.8 (Waring *et al.* 2015). This makes sense from a metabolism perspective as a more lipophilic compound produces more metabolites with a greater number of metabolizing enzymes involved. Thus, the probability for the production of reactive metabolites and for drug interactions increases with increasing compound lipophilicity. Very lipophilic compounds should have high potency (i.e., low daily dose) to avoid adverse events (Chen *et al.* 2013). Fortunately, the activity to the intended indication tends to increase by increasing molecular weight and lipophilicity (Perola 2010). However, selectivity has the opposite tendency to decrease by increasing lipophilicity (Leeson and Springthorpe 2007). With high lipophilicity, the likelihood to bind to multiple targets increases and as a result pharmacologically-based toxicity (Type A2 toxicity, see 2.8.2) may become an issue. The aim is to find an acceptable chemical compromise between lipophilicity, activity, selectivity and metabolic properties, and the trick is to increase potency without increasing too much lipophilicity.

### *2.2.2 Drug metabolizing enzymes*

A study of the top 200 prescription drugs reveals that the majority of the drugs have metabolism as the main clearance mechanism (73%), whereas 24% have renal clearance and only 3% biliary clearance as the main mechanism (Williams *et al.* 2004, Wienkers & Heath 2005). Generally, the majority of drugs are lipophilic compounds requiring biotransformation(s) to a more hydrophilic form before they can be excreted into bile or urine. Metabolism usually increases the molecular weight, polar surface area (PSA) and solubility of the molecule, and all these decrease the permeability of membranes. Consequently, transporters are needed to carry the metabolites out from the hepatocyte (or enterocyte) and excrete them. If the polar surface area of the metabolite – or a hydrophobic drug – is high, they are transported to bile, and if PSA is low, they are transported to urine (Wu & Benet 2005, Zamek-Gliszczynski *et al.* 2006a, Benet 2010, Di *et al.* 2013a).

The cytochrome P450 enzymes (CYP) metabolize 73% of drugs cleared through metabolism as primary mechanism before they are excreted or subjected to further metabolism. UDP-glucuronosyltransferases (UGT) are responsible for the first-line metabolism of 15% of drugs, esterases (hydrolases) for 9% and other enzymes for 3% of drugs (Williams *et al.* 2004). The most important CYP enzyme is CYP3A4 which is responsible for almost half of the CYP catalyzed oxidations as main route. About 95% of all CYP-mediated metabolism as main route is catalyzed by only five CYP enzymes: 1A2, 2C9, 2C19, 2D6 and 3A4 (Williams *et al.* 2004). There are a total of 57 CYP enzymes (Guengerich 2008). When 4,192 oxidoreductase metabolic reactions of 860 drugs (each drug potentially undergoing multiple reactions) and their respective metabolic enzymes were examined, the CYP superfamily accounted for more than 95% of all the reactions documented (Rendic & Guengerich 2014). The same five CYP enzymes account for 68% of all oxidoreductase reactions. The other notable oxidoreductase families were microsomal flavin-containing mono-oxygenase (FMO, 2% of all reactions), monoamine oxidase (MAO, 1%) and aldo-keto reductase (AKR, 1%) (Rendic & Guengerich 2014). For drugs containing polar heterocycles or aza-aromatics used to reduce lipophilicity, increase metabolic stability or to achieve special binding interactions, aldehyde oxidases (AO) are of elevated importance as a class of oxidoreductases (Zientek & Youdim 2014, Sanoh *et al.* 2015).

The liver is responsible for most of the metabolism affecting drugs, but there are significant levels of drug-metabolizing enzymes in other organs as well. Especially the small intestine has high levels of CYP and UGT enzymes that correspond to the first-pass metabolism of drugs and thus to the overall bioavailability of orally administered drugs (Paine *et al.* 2004). Human UGT enzymes 1A1, 1A3, 1A4, 1A6, 1A9, 2B7, 2B10 and 2B15 are the most important ones in hepatic drug glucuronidation and UGT1A10 has an important role in the intestine (Oda *et al.* 2015). Esterases catalyze hydrolysis of amides, esters and thioesters, and carboxylesterases 1 and 2 (CES1 and CES2) are primarily responsible for the hydrolysis of drugs (Oda *et al.* 2015). Table 1 summarizes the relative abundances of CYP isoforms found in the liver (Rowland-Yeo *et al.* 2004) and small intestine (Paine *et al.* 2006) together with the proportion of drugs metabolizing through these CYPs (Williams *et al.* 2004).

**Table 1. Drug metabolizing CYP enzymes, their relative abundance in the liver and intestine, and the proportion of drugs metabolized via CYP enzymes as the main clearance mechanism.**

| Drug metabolizing CYP isoform | Relative CYP abundance in the liver (Rowland-Yeo *et al.* 2004) | Relative CYP abundance in the small intestine (Paine *et al.* 2006) | Proportion of drugs metabolized (Williams *et al.* 2004) |
|---|---|---|---|
| CYP1A1 | | 8% | |
| CYP1A2 | 12% | | 9% |
| CYP2A6 | 8% | | |
| CYP3A4 | 36% | 59% | 46% |
| CYP3A5 | | 20% | |
| CYP2B6 | 3% | | 2% |
| CYP2C8 | 6% | | |
| CYP2C9 | 17% | 11% | 16% |
| CYP2C19 | 3% | 1% | 12% |
| CYP2D6 | 2% | 1% | 12% |
| CYP2E1 | 14% | | 2% |

Concerning the enzyme abundance data in Table 1, it has been pointed out that the measurements of protein contents are not too comprehensive as liver CYP abundances are measured from 42–241 liver samples depending on the CYP enzyme and intestine measurements are made only from 3–31 small intestine samples (Galetin *et al.* 2010). CYPs 3A4 and 3A5 are usually bundled together in the literature as the contribution of 3A5 to drug metabolism is not fully understood, and the same goes for the CYP2C family, as 2C8, 2C9 and 2C19 are sometimes summed together. It is important to notice the significance of two minor abundance CYP enzymes that provide major clearance mechanism for almost one in every four drugs cleared via CYP metabolism, namely CYP2C19 and CYP2D6. They are both genetically polymorphic, especially CYP2D6, and polymorphism may lead to highly reduced or elevated circulating concentrations of the drugs (or metabolites) known to metabolize via these enzymes. The reduction/elevation of the drug/metabolite concentration depends on the specific polymorphism. As a consequence, toxic events may emerge due to too high a concentration of a drug (or a metabolite), or therapeutic loss of response may happen due to too low drug concentrations (Johansson & Ingelman-Sundberg 2011 and references cited therein). According to Rendic & Guengerich (2014) 20% of the oxidoreductase reactions of drugs under development are still produced by CYP2C19 and CYP2D6 despite the polymorphism issue and the avoidance of their substrates during the lead optimization. It is probable that

future medications will frequently be accompanied by genetic testing for polymorphism to aid selection of individually suitable drugs and therapeutic doses (Singh *et al.* 2011).

One should keep in mind, however, that the quantitative amount of total drug metabolizing CYP enzymes (per mg protein) is about six times higher in the liver than in the intestine (Paine *et al.* 2006, Rowland-Yeo *et al.* 2004) and additionally, the weight of the liver is more than two-times that of the small intestine, 1,500 g vs. 700 g on average (Lin *et al.* 1999). Thus, the metabolic capacity of the liver is at least 15-fold greater compared to that of the small intestine. The abundance of CYP enzymes and transporters varies in different parts of the intestinal wall. The bioavailability can be increased for drugs with slow release formulation by escaping intestinal metabolism or efflux transporters of the early parts of the intestine (Jamei *et al.* 2009). Concerning other human organs, the kidney can have substantial contribution to the metabolism (e.g. glucuronidations) of some drugs (Knights *et al.* 2013), while skin has significant phase II metabolism potential (glucuronidation, sulfation, N-acetylation, catechol methylation and glutathione conjugation) for topical and transdermal applications as well as for cosmetics (Manevski *et al.* 2014). Target-specific metabolism (e.g. metabolism in brain or lung) may sometimes play an important role in the elimination (and efficacy) of drugs (Foti *et al.* 2015).

The gastrointestinal microbiota is a major non-human organ whose contribution to the human drug metabolism has been overlooked. There are 100 trillion microbes the human gastrointestinal tract and they are known to metabolize at least 30 marketed drugs. Most of the bacterial metabolic reactions are different from typical reactions provided by human metabolizing enzymes (e.g. reductions) and thus the metabolites may appear as surprises rather late in the development and produce pharmacological or toxicological effects not anticipated for the developed drug or its metabolites (Sousa *et al.* 2008). The microbiota may also prolong the duration of drug action by enterohepatic circulation, in which inactive conjugated drug (sulfate or glucuronic acid) excreted to bile is deconjugated by the bacteria, reabsorbed and returned to the circulation (Gao *et al.* 2014).

### *2.2.3 Metabolism reactions*

Each compound can undergo several streams and steps of metabolism, as can be seen from the massive analysis of 6,767 metabolic reactions from over 1,171

substrates (medicinal and other xenobiotic compounds) extracted from three journals (Chemical Research Toxicology, Drug Metabolism and Disposition and Xenobiotica) published between 2004 and 2009. The results are summarized in Table 2 (Testa *et al.* 2012). The average number of was six metabolites per substrate. First generation metabolites accounted for 42% of all metabolites, 32% were second generation and 26% third or later generation. Interestingly, over 70% of the 201 (3%) pharmacologically active metabolites found were first generation, whereas for 473 (7%) toxic or potentially toxic (chemically reactive) metabolites the distribution of generations was practically identical to that of all metabolites, indicating that reactive metabolites can be formed almost equally at any generation (Testa *et al.* 2012).

Redox reactions accounted for 57% of all metabolites, esterases for 10% and conjugations for 33%. A total of 40% and 14% of the reported 6,767 metabolites were produced by CYP and UGT enzymes, respectively. However, for the first generation metabolites the proportions were 58% and 11% for CYPs and UGTs, respectively. So, even if the majority of metabolically cleared compounds are first-line substrates for CYP enzymes, the majority of end-products are formed by enzymes. The later generation the metabolite is the more important other than CYP enzymes are in its production. Actually, 60% of third and further generation metabolites are produced by other than CYP or UGT enzymes! The proportions of CYP-mediated oxidations were 33% and 23% in the second and third generations, respectively (Testa *et al.* 2012).

The low amount of active metabolites is not surprising as most of the substrates of medicinal interest were investigational drugs and their metabolite activity is normally not known. However, the reactions and enzymes producing active metabolites are not surprising: 37% ester hydrolyses and 36% by C-hydroxylations (Testa *et al.* 2012). Simple hydroxylated metabolite may well retain the target affinity and pharmacological activity (Fura 2006), whereas ester hydrolysis is a major approach to cleave promoieties from prodrugs to gain pharmacologically active metabolites (Rautio *et al.* 2008).

**Table 2. Different drug metabolism reactions, their responsible enzyme superfamilies and the relative abundance of produced metabolites (Redrawn from Testa *et al.* 2012).**

| Drug metabolism reaction | Responsible enzyme superfamily | % |
|---|---|---|
| Redox reactions | | |
| Oxidation of C-$sp^3$ | CYPs | 20.6 |
| Oxidation of C-$sp^2$ and C-sp | CYPs (AOR, XOR) | 13.9 |
| CHOH <-> C=O and C=C -> COOH | Dehydrogenases | 8.3 |
| Oxidation of $R_3N$ (tertiary amines) | CYPs, FMOs | 1.3 |
| Reduction of N-oxides | Reductases | 0.1 |
| Oxidation of NH or NOH | CYPs (FMOs) | 1.4 |
| Reduction of $NO_2$, N=O or NOH | Reductases | 2.6 |
| Oxidation to quinones or anologs | Peroxidases, CYPs | 3.4 |
| Reduction to quinones or anologs | Reductases | 0.6 |
| Oxidation of S atoms | CYPs, FMOs | 2.6 |
| Reduction of S atoms | Reductases | 0.2 |
| Other redox reactions | Reductases, other OXRs | 1.7 |
| Hydrolytic reactions | | |
| Hydrolysis of esters, lactones or inorganic esters | Esterases | 3.8 |
| Hydrolysis of amides, lactams or peptides | Esterases | 1.7 |
| Hydration of epoxides | Esterases | 1.6 |
| Other reactions of hydration or dehydration | Esterases, non-enzymatic hydrolyses or (de)hydrations | 2.8 |
| Conjugation reactions | | |
| O-Glucuronidation or glycosylations | UDP-Glucuronosyltransferases | 12.7 |
| Other gluguronidations (N, S or C) | UDP-Glucuronosyltransferases | 1.4 |
| Sulphonations | Sulphotransferases | 4.8 |
| Conjugations with glutathione and their sequels (incl. reductions) | Glutathione-S-transferase and associated enzymes | 8.0 |
| Acetylations | Acetyltransferases | 1.5 |
| CoAs-ligation and aminoacyl conjugations | Acyl-CoA ligases and associated enzymes | 1.8 |
| Methylations | Methyltransferases | 2.2 |
| Other conjugations ($PO_4$, C=O) | Other transferases or non-enzymatic reactions | 0.9 |

Potentially reactive or toxic metabolites are formed at any generation of metabolites as previously described. By far the most important reactions are oxidations to quinones, quinonimines, quinonimides and quinonedi-imides (41% of all alerting metabolites). Together oxidations account for about 85% of all toxic or reactive metabolites. Major contributions are due to aromatic hydroxylations

15% (epoxides), N-oxidations 8% (hydroxyl amines and nitroso compounds) and S-hydroxylations 9% (sulfenic acids) (Testa *et al.* 2012).

## 2.3 Drug-drug interactions

When medications are administered concomitantly, drug-drug interactions (DDI) may happen, meaning that one drug (perpetrator) alters the pharmacokinetics of another drug (victim) causing a change of exposure of the victim drug and its metabolites (Bode 2010, Prueksaritanont *et al.* 2013). Sometimes the perpetrator is not the drug but its metabolite (Isoherranen *et al.* 2009). The most studied classes of DDIs are CYP enzyme inhibition and induction, but interactions may involve other metabolizing enzymes as well as transporters. Unlike other drug metabolizing enzymes and transporters most of the CYP enzymes have relatively specific substrates, inhibitors and inducers. These can be used during drug discovery and development to study the potential of lead molecules and candidate drugs to cause or be victims to DDIs (Pelkonen *et al.* 2008a).

### *2.3.1 Enzyme inhibition*

In enzyme inhibition, the perpetrator drug inhibits the action of an enzyme responsible for the metabolism of the victim drug causing an increase of the exposure of the victim drug. This may lead to potentially serious side effects unless there are compensatory clearance mechanisms. The most important drug metabolizing enzyme family is the CYPs, as they are generally the rate-limiting enzymes in drug clearance. However, UGT enzyme inhibition is an emerging field especially for the compounds having direct glucuronidations as the major metabolic clearance pathway. Human liver microsomes and recombinant CYP/UGT enzymes are the most frequently used systems to study CYP/UGT inhibition, whereas human hepatocytes are not that common a system due to a more complex and time consuming setup (Pelkonen *et al.* 2005, Pelkonen *et al.* 2008b). In many cases the drug candidate is metabolized extensively by other enzymes than CYPs and the use of simplified inhibition systems may give biased results. Such system-dependent differences in the CYP inhibition would call for additional examination with human hepatocyte system (Parkinson *et al.* 2010). With careful selection of CYP-specific victim drugs and their concentrations they can be co-incubated (*in vitro* cocktail) together with one potential perpetrator (NCE) in human liver microsomes. The cocktail approach is used early in the

drug development to find in a robust, automated and time-saving manner the potential of CYP inhibition for new chemicals towards all important CYPs (Turpeinen *et al.* 2005, Turpeinen *et al.* 2006, Tolonen *et al.* 2007, Liu *et al.* 2014). Similar *in vitro* cocktail approaches have also been developed to screen UGT enzyme inhibition in human liver microsomes (Joo *et al.* 2014, Gradinaru *et al.* 2015).

Mostly, CYP inhibition is instant and reversible, as the inhibitor binds non-covalently to the CYP enzyme. Time-dependent inhibition (TDI) happens when there is a clear time lag before the inhibition action starts. Time-dependence can be studied by pre-incubation with (presumed) inhibitor before adding the victim drug. If the pre-incubation enhances the inhibition, TDI is involved. The mechanisms behind TDI include formation of a perpetrator metabolite acting as reversible inhibitor to a CYP or formation of a perpetrator reactive intermediate (metabolite) that binds covalently and irreversibly to the CYP enzyme catalyzing the formation. In the more serious latter case (also referred to as mechanism-based inhibition, MBI) the inhibition lasts longer and increases after multiple dosing. There is an industry consensus as to how to conduct TDI studies on human liver microsomes (Grimm *et al.* 2009), but some studies indicate that better predictions may be obtained by using cryopreserved or primary hepatocytes (Xu *et al.* 2009, Chen *et al.* 2011).

In transporter inhibition, the perpetrator inhibits the operation of a transporter (or transporters) involved in intestinal, hepatic or renal uptake, or in intestinal or hepatic efflux of a victim drug or its metabolite. Depending on the inhibited transporter(s) this may lead to adverse events due to prolonged circulation or accumulation to organs of the victim drug or its metabolites, or to therapeutic failure. As described by Bode (2010) complex interactions may involve both CYP enzymes and transporter and they are practically impossible to predict based on *in vitro* observations. The kinetics of transporter-related interactions is not thoroughly understood, but simple competition mechanism is commonly assumed (Prueksaritanont *et al.* 2013). The interaction data derived from *in vitro* transporter models have high inter-laboratory variation and dependence on the systems and methodology used. As an individual transporter alone has usually less than three-fold clinical DDI effect, it is questioned if comprehensive transporter studies as a routine are justifiable (Prueksaritanont *et al.* 2013). Recently, an *in vivo* cocktail of four transporter probe drugs was proposed for the simultaneous clinical investigation of inhibition of P-gp, BCRP, OATP and OCT (Ebner *et al.* 2015).

CYP inhibition related DDIs do not only involve the liver, but also the intestine. Enterocytes have a high content of especially CYP3A which should be taken into account when quantitatively predicting overall DDI risks (Galetin *et al.* 2010). A method for predicting intestinal drug-drug interactions involving CYP3A4 and efflux transporter P-glycoprotein (MDR1, P-gp) has been proposed (Tachibana *et al.* 2009). However, Kosugi *et al.* (2012) noticed in a retrospective study of 35 reported clinical DDI cases that for CYP3A4 the DDIs were overestimated, if intestinal DDI was taken into account.

### *2.3.2 Enzyme induction*

Enzyme induction by perpetrator creates an opposite effect compared to inhibition when the amount of metabolizing enzyme increases. As a result the extent of victim metabolism increases leading to increase of metabolite exposure and decrease of victim drug exposure, which may potentially cause therapeutic failure. Enzyme induction takes some time to evolve as the mechanism involves activation of nuclear receptors CAR and PXR which regulate the expression of their target genes affecting the levels of CYP enzymes, conjugation enzymes (UGTs and sulfotransferases) and transporters (Pelkonen *et al.* 2008a). With some chemicals the nuclear receptor activation can be very selective towards certain CYP enzyme induction (Kühlbeck *et al.* 2011).

Because of the mechanism, the *in vitro* methods to study DDIs caused by CYP enzyme induction have to be performed with hepatocytes or cell lines expressing the needed nuclear receptor activation pathway (Hewitt *et al.* 2007). The human immortalized hepatoma cell line HepaRG is a good example of a cell line expressing all major drug metabolizing enzymes and transporters as well as nuclear receptors (Guillouzo *et al.* 2007). The functionality and inducibility of HepaRG has been proved (Turpeinen *et al.* 2009, Anthérieu *et al.* 2010). Like intestinal CYP enzyme inhibition also intestinal CYP enzyme induction may happen, and sometimes the intestinal induction may be over 8-fold greater than the induction in the liver measured by comparison of induction effect after oral and i.v. administration (Galetin *et al.* 2010). An *in vitro* cocktail approach to study induction potential of NCEs early in the discovery phase has been developed for CYP enzymes 1A2, 2B6, 2C9 and 3A4 using cryopreserved human hepatocytes (Gerin *et al.* 2013).

### 2.3.3 Metabolizing CYP enzyme identification

By identifying the CYP enzymes responsible for the metabolism of the candidate drug it is possible to evaluate the probability of the candidate becoming a victim of a DDI. The identification can be achieved by incubating the study compound with human liver microsomes or hepatocytes and adding CYP-specific inhibitors one-by-one into the system (Pelkonen *et al.* 2005, Pelkonen *et al.* 2008b). Observation of which metabolite disappears (compared to incubation without inhibitor) gives indirect information about the corresponding CYP enzyme. A direct approach utilizes incubation of the study compound with single recombinant CYP enzymes. Observation of a metabolite appearing in incubation gives evidence of the involvement of the respective CYP enzyme (Pelkonen *et al.* 2005, Pelkonen *et al.* 2008b). It is advisable to use both direct and indirect approaches to safely state that a certain metabolite is formed by a certain CYP enzyme. The above-mentioned approaches also apply to UGT enzymes, as they have the most important recombinant enzymes available. However, the known inhibitors lack specificity, which has to be taken into account when using the indirect method.

### 2.3.4 Regulatory guidance for drug-drug interactions

Both FDA and EMA released guidance for the investigation of drug interactions in 2012 (U.S. Department for Health and Human Services 2012 (later FDA 2012), European Medicines Agency 2012 (later EMA 2012)) to support market authorization. Even though the FDA guidance is a draft guidance with nonbinding recommendations, the FDA reviewers have already referenced it when giving instructions to drug developers (Prueksaritanont *et al.* 2013). While both guidances were released almost simultaneously and have quite a similar concept based on the same state-of-the-art science, they contain marked differences. Most importantly, according to the EMA the evaluation of CYP and transporter inhibition (and triggering need for *in vivo* interaction trials) should be based on unbound plasma concentration, whereas the FDA favors the use of total plasma concentration (FDA 2012, EMA 2012). This poses a challenge for drug developers as the same data can be predicted differently with different regulatory specifications and the extent of interaction potential may be changed (Kosugi *et al.* 2012, Prueksaritanont *et al.* 2013). A comparison between cryopreserved human hepatocytes suspended in human plasma (total plasma) and in protein-free

hepatocyte maintenance medium (unbound plasma) shows that the total plasma method predicts 89% of 63 reported clinical interactions studies with 17 different CYP3A, CYP2C9 or CYP2D6 inhibitors correctly, while the protein-free method predicts only 59% correctly (Mao *et al.* 2011, Mao *et al.* 2012). Moreover, the total plasma method predicted both reversible and time-dependent interactions. On the other hand, another retrospective study of 26 GSK compounds having both clinical DDI data and consistent microsomal data, points out prediction superiority for unbound concentrations over the use of total concentrations: average prediction 91% vs. 52% within two-fold observed AUC, respectively (Shardlow *et al.* 2011). Additionally, considering intestinal contribution to DDI, the study shows similar average prediction power for the unbound method whether or not the intestinal contribution is left out (93% vs. 91%), and significantly poorer prediction power for the total concentration method if the intestinal DDI contribution was left out (35% vs. 52%) (Shardlow *et al.* 2011).

In addition to CYP enzyme inhibition, EMA also requires studying inhibition of UGT1A1 and UGT2B7, if one of the major elimination pathways is glucuronidation of the parent drug. Both UGTs are important in endobiotic metabolism (1A1: bilirubin glucuronidation, 2B7: glucuronidations of hormones and fatty acids). In the area of transporters, the EMA requires inhibition study of the bile salt export pump (BSEP), which the FDA does not require (EMA 2012, FDA 2012). Again, BSEP has important endogenous function likely to influence the requirement, and the inhibition of BSEP has been associated with cholestatic drug-induced liver injury (Garzel *et al.* 2014, Dawson *et al.* 2012). EMA also provides clearer guidance concerning when to conduct DDI studies with the main metabolites and emphasizes that all *in vitro* interaction studies should be performed before the onset of Phase II (EMA 2012). FDA introduced transporter interaction decision trees in 2008 (Zhang *et al.* 2008) and both guidances include now several helpful decision trees for drug developers.

Concerning *in vitro* CYP induction studies, the end point should be the mRNA level compared to control, not levels of probe metabolites as enzyme activity measure. Induction studies should be performed on cultured fresh or cryopreserved hepatocytes, not on e.g. HepaRG cell line, which can, however, provide supportive information. According to EMA, the proper study of mixed-mode inhibition/induction always requires *in vivo* interaction trial. Neither agency recommends *in vitro* induction studies for transporters. (EMA 2012, FDA 2012). The analysis of NDA (new drug application) reviews for NMEs approved by FDA

in 2013 shows general consistency with the current recommendations (Yu *et al.* 2014).

### 2.3.5 Metabolites causing CYP enzyme inhibition

Concerning drug metabolites, the FDA recommends considering both potential enzyme and transporter DDI studies when a metabolite is present at ≥25% of the parent drug AUC, whereas the EMA requires the same for metabolites present both at ≥25% of the parent drug AUC and ≥10% of exposure of total drug-related material (EMA 2012, FDA 2012). In the case of parent being an inhibitor and *in vivo* DDI study is conducted the metabolite contribution will be automatically included at a clinically relevant level unless there are populations where metabolites of such parent could be present at considerably higher levels (Yu and Tweedie 2013).

A literature review of 129 CYP inhibiting drugs revealed that a majority of them (82%) had confirmed circulating metabolites and the 21 most potent inhibitors all had reported circulating metabolites (Isoherranen *et al.* 2009). Worryingly, only 34 circulating metabolites were investigated *in vitro* for inhibitory potential and for 14 of them either parent drug or metabolite (or both) were identified as MBIs (Isoherranen *et al.* 2009). Later the same group continued the study with 72 inhibitory compounds having parent and circulating metabolite AUC data available. In the resulting 120 parent-metabolite AUC data sets, in over 80% of the cases the metabolite AUC was ≥25% of the parent AUC (Yeung *et al.* 2011). However, only 29 metabolites of 21 drugs were characterized *in vitro* for their inhibitory potency: in 13 cases the *in vitro* potential was greater with the metabolite than with the parent and only in 8 cases the metabolite was predicted not to contribute to the inhibition (Yeung *et al.* 2011). Although the metabolites were observed as likely to contribute to *in vivo* CYP enzyme inhibition in most of the cases, there were only three inhibitors whose inhibitory potential would not have been predicted based on the parent compound *in vitro* data only (out of 21 drugs whose metabolites were tested *in vitro*) (Yeung *et al.* 2011).

In a Pfizer survey of their 33 clinical compounds having altogether 115 circulating metabolites over half of the metabolites (60) had exposure levels greater than 25% of the parent drug. However, only 14 metabolites of 12 parents were tested with synthetic standards: no clinically relevant inhibitory DDI were observed (Callegari *et al.* 2013). Additionally, Callegari *et al.* (2013) propose a static method to evaluate DDI potential and subsequent need for *in vivo* DDI

study of a major metabolite having ≥25% of the parent drug AUC. The model is based on *in vivo* measured Parent $C_{max}$ (from human PK), Metabolite/Parent AUC ratio (from human mass balance study) and *in vitro* measured $K_i$ of the metabolite. In case where $K_i$ is not available for the metabolite, $4*K_i$ of the parent is used (Callegari *et al.* 2013). As metabolites are usually more polar than the parent and tend to have less affinity to drug metabolizing enzymes, Yu and Tweedie (2013) propose that only metabolites less polar than parent having ≥25% of the parent drug AUC should be tested *in vitro* for DDI potential. More polar metabolites should be tested only if their AUC ≥100% of the parent (Yu & Tweedie 2013). However, major metabolites with structural alerts for MBI, which are not present in the parent structure, should be evaluated on a case-by-case basis (Yu and Tweedie 2013, Yu *et al.* 2015). A common metabolic reaction possessing structural alert to cause MBI is N-dealkylation of secondary/tertiary amines to form primary amines that can further metabolize to nitroso intermediates and inactivate corresponding CYP enzyme (Orr *et al.* 2012).

A group of metabolism scientists from 18 pharmaceutical companies analyzed literature for drug interactions of the 137 most frequently prescribed drugs to assess the risk of metabolites as the sole contributor to CYP enzyme inhibition (Yu *et al.* 2015). The risk is low as only metabolites of 5 drugs (3.6%) were likely to be the sole perpetrators. For majority of drugs, circulating metabolites seem not to be important in causing *in vivo* DDIs. However, many metabolites alter the time course and magnitude of inhibitory DDIs (Varma *et al.* 2015). Additionally, 10 of the 19 MBI causing drugs with known mechanism have circulating metabolites known to cause CYP inactivation (VandenBrink & Isoherranen 2010).

## 2.4 Prediction of pharmacokinetics

Pharmacokinetics, the fate of a drug from administered dose to elimination outside the body, is a complex interplay of drug absorption, distribution, metabolism and excretion involving active and passive events, drug metabolizing enzymes and drug transporters, drug-drug and drug-food interactions, binding of drug to proteins and cellular systems, and so on. Pharmacokinetics is explaining what is happening to a drug in the body, and when and how it comes out from the body.

### 2.4.1 The role of drug transporters

In addition to numerous different drug metabolizing enzymes there are over 20 clinically important transporters that affect the pharmacokinetics of drugs and metabolites in absorption, disposition and excretion. They locate mainly in the intestine, liver, kidney and blood-brain barrier (Giacomini *et al.* 2010). The organ location of the most important drug transporters and the operational directions where they transport the drugs and metabolites have been well described (Giacomini *et al.* 2010, König *et al.* 2013, Nigam 2015). Since the introduction of transporter drug-drug interactions to regulatory interest and subsequently to guidances (FDA 2012, EMA 2012), the understanding of the interplay between drug transport and drug metabolism in the combined kinetics of absorption, disposition and elimination of drugs and their metabolites has increased (König *et al.* 2013, Hochman *et al.* 2015). However, the understanding of the endogenous roles of transporter proteins is limited (Nigam 2015).

Drug metabolism generally increases polarity and metabolic stability compared to parent drugs. However, these properties together with generally lower membrane permeability mean that their disposition is likely to involve transporters, and thus, metabolites might cause drug transporter interactions (Zamek-Gliszczynski *et al.* 2014). Metabolite-transporter interactions are very challenging to explore for several reasons. First of all, it is difficult to distinguish relative contribution of parent drug and metabolite(s) in drug interactions (Schuetz *et al.* 2014). Secondly, contrary to drug metabolizing enzymes, there are only a small number of specific substrates and inhibitors for most of the transporters (Brouwer *et al.* 2013) because they may have multiple binding sites and large binding pockets (Aller *et al.* 2009). The third reason is the complex clinical interplay between the uptake and efflux transporters, and metabolizing enzymes in different organs (Bode 2010). The *in vitro* investigation methods for transporter induction are not well established even though P-glycoprotein activity in Caco-2 cells is inducible through cell line transfection (Korjamo *et al.* 2006).

Generally, lipophilic metabolites permeate passively to sinusoidal blood and circulation from the liver, whereas hydrophilic metabolites, especially conjugates like glucuronides, sulfates and glutathione adducts, are actively carried immediately after formation to bile (apical/canalicular transport) and then directed to feces, or to sinusoidal blood (basolateral transport) and circulation, and eventually excreted into urine (Zamek-Gliszczynski *et al.* 2006a). It remains unclear why certain metabolites are excreted apically from the liver and some

basolaterally, i.e., why some metabolites circulate while others do not (Zamek-Gliszczynski *et al.* 2014). For biliary excreted metabolites, enterohepatic circulation may take place for intact oxidized metabolite or for conjugated drug or oxidized metabolite (after deconjugation by intestinal microbiota). In some cases, practically the whole exposure of conjugated metabolites is found from the feces and none of them circulate (Zamek-Gliszczynski *et al.* 2006a), but for nonconjugated metabolites there are only a few of such reports (Zamek-Gliszczynski *et al.* 2013, Li *et al.* 2014.). Extensive enterohepatic circulation of metabolites may lead to hepatic accumulation and hepatotoxicity.

### *2.4.2 Prediction of clearance*

Prediction of metabolic clearance of a drug is quite a well understood and mature area of *in vitro* - *in vivo* extrapolation (IVIVE) especially concerning CYP-mediated metabolism (Houston 2013). Metabolic clearance affects drug half-life and oral bioavailability, which are important factors for the establishment of dose and dosing regimen. Drug developers start measuring the metabolic clearance early on in the drug discovery process aiming to find and develop further compounds having suitable metabolic clearing characteristics for once daily oral dosing.

The initial metabolic clearance measurement is based on parent drug depletion when incubated with human liver microsomes and appropriate cofactors for CYP and UGT mediated metabolism. Thus, the basic setup has the limitation of not involving all potential metabolism routes. Other limitations are the short lifetime of enzymatic activity and general underprediction of hepatic intrinsic clearance from microsomal data. However, due to a rather simple and inexpensive setup, relative ease of extrapolation and ability to rank compounds with high throughput, the use of liver microsomes is likely to remain the first-line choice for early metabolic clearance measurement. With appropriate analytical instrumentation the major metabolites of first and second generation catalyzed by CYP and UGT enzymes can be detected and their structures tentatively elucidated (Pelkonen *et al.* 2005, Pelkonen *et al.* 2008b).

Later during the drug discovery and development process a more comprehensive system is needed to characterize metabolic and hepatic clearance. The role of human hepatocytes has been emphasized to a great extent (Hewitt *et al.* 2007, Soars *et al.* 2007, Li 2007, Gómez-Lechón *et al.* 2007), as all the drug metabolizing enzymes are present, as well as uptake and efflux transporters.

Plated and cultured human hepatocytes are the golden standard in metabolic clearance and metabolite formation studies, even though the levels of drug metabolizing enzymes may decrease during cultivation (Pelkonen *et al.* 2008b). Commercial availability of pooled cryopreserved hepatocytes has increased their use. There is general consensus that the enzyme activities are preserved under cryopreservation and they can be routinely used for the evaluation of drug metabolism related processes (Li 2007). The incubation time with cryopreserved hepatocytes is longer than with microsomes (4 h vs. 1 h), but the resulting hepatic clearance is underpredicted compared to microsomes (Brown *et al.* 2007, Stringer *et al.* 2008). However, it can be corrected to the same level by better consideration of fraction unbound in hepatocytes and microsomes (Hallifax & Houston 2006, Kilford *et al.* 2008). Still, the relative underprediction prevails for high clearance compounds (Hallifax *et al.* 2010). This was also studied with human liver microsomes and suspended hepatocytes from the same four donors, and a four-fold average underprediction for high clearance compounds was found (Foster *et al.* 2011). Concerning microsomal prediction for highly protein bound compounds, considerable tailoring of intrinsic clearance parameters is presented in terms of drug ionization state and which proteins they actually bind (Poulin *et al.* 2012). The same method was not shown to offer significant improvement in clearance prediction for a larger set of compounds regardless of whether the data came from microsomes or hepatocytes (Hallifax and Houston 2012).

The proportion of slowly metabolizing compounds has increased in the drug discovery process partly due to increased screening of metabolic clearance and biased selection towards low clearance compounds. In many cases the clearance cannot even be predicted as there is no or too low a turnover (depletion) of the parent compound. To solve this, a hepatocyte relay incubation method was developed and a two-fold difference between *in vitro* and *in vivo* intrinsic clearance was achieved for most compounds (Di *et al.* 2012, Di *et al.* 2013b). The difference is similar compared to what is generally achieved for high and moderate clearance compounds with hepatocytes. In the relay method, the supernatant is removed and added to a fresh plate of cryopreserved hepatocytes every 4 hours and standard procedure includes five relay cycles (Di *et al.* 2012). Another approach is HepatoPac, a micropatterened hepatocyte-fibroblast coculture system continuously usable for metabolism incubations even up to 7 days (Khetani & Bhatia 2008, Wang *et al.* 2010, Chan *et al.* 2013). The results (Chan *et al.* 2013) are comparable to Di *et al.* (2013b), but the experimental setup is much more complicated and laborious (Khetani & Bhatia 2008). Additionally,

cryopreserved HepaRG cells have shown to provide hepatic clearance predictions quantitatively comparable to cryopreserved hepatocytes (Zanelli *et al.* 2012).

For drugs anticipated to be hepatic transporter substrates, hepatocyte media loss assay may provide more reliable clearance prediction than conventional hepatocyte incubation, even though the method only provides 50% accuracy to identify the actual transporter involvement (Jigorel & Houston 2012). The prediction of intestinal clearance can be done based on permeability and human liver microsomal clearance normalized to tissue-specific (intestine-liver) CYP3A content (Gertz *et al.* 2010). Generally good predictions are obtained for compounds with low to moderate intestinal extraction, but not for high extraction compounds (Gertz *et al.* 2010). For compounds metabolizing mainly through CYP3A4, incubation with rat intestinal S9 fraction has proved to give reliable prediction of intestinal clearance, but for compounds additionally metabolizing through CYP2C enzymes, overprediction of intestinal extraction is observed, especially for low permeability compounds (Karlsson *et al.* 2013).

### *2.4.3 Physiologically based pharmacokinetic modeling*

Physiologically based pharmacokinetic (PBPK) modeling is a widely used simulation tool to predict pharmacokinetics and drug interactions of drug candidates in humans during clinical development as well as to give mechanistic insight into compound ADME properties (Pelkonen *et al.* 2011, Rostami-Hodjegan 2012, Jones *et al.* 2012, Jones *et al.* 2015). It has regulatory acceptance to illustrate the pharmacokinetic behavior of a compound and even to replace certain clinical studies (e.g. drug-drug interactions) provided that input data is of high quality and the simulation addresses a specific question (Huang 2012, Zhao *et al.* 2011, Sinha *et al.* 2014). According to FDA, since 2008, drug developers have started to include PBPK modeling and simulations regularly into IND applications and NDAs, and even FDA reviewers frequently conduct PBPK simulations to support the reviews (Zhao *et al.* 2011). The FDA drug interaction draft guidance puts strong emphasis on the use of PBPK models and plans to use it in DDI studies (FDA 2012). Additionally, the FDA has proposed a workflow for determining TDI potential of a new drug utilizing the PBPK model (Vieira *et al.* 2012) and shown the utility of the PBPK approach in quantitative prediction of complex drug-drug-disease interaction (Grillo *et al.* 2012).

The PBPK model can evaluate the effects of intrinsic (e.g. age, race, disease, organ dysfunction, pregnancy, gender, genetics) and extrinsic (e.g. concomitant

medications, alcohol use, smoking, environment, diet, herbs) factors, alone or in combinations, to enable numerous 'what if' simulations of pharmacokinetics and drug exposure to help explain and design clinical trials and drug interaction studies. The general process to build and refine the PBPK model is the following (Zhao *et al.* 2011):

1. Identification and quantification of clearance pathways (main metabolic enzymes, renal and biliary excretion) based on *in vitro* and *in vivo* data.
2. Building the model based on IVIVE and incorporation of drug and its physicochemical and physiological parameters.
3. Comparison of initial simulated pharmacokinetics (plasma concentration-time profile) to *in vivo* first-in-human (FIH) studies.
4. Model refinement based on sensitivity analysis and/or comparison to *in vivo* pharmacokinetics.
5. Simulation of PK profiles under various scenarios. Refinement of the model when additional *in vivo* data is obtained.

Besides being used in the clinical phase of drug development PBPK has recently gained popularity also in the discovery phase in the prediction of absorption and bioavailability, tissue distribution and clearance (Rostami-Hodjegan 2012, Chen *et al.* 2012, Shaffer *et al.* 2012). However, one of the main challenges is the incorporation of active transport processes to the models, which affect especially the renal and biliary clearance of drugs and metabolites (Smith 2012, Jones *et al.* 2012) that cannot be easily studied *in vitro*. Application of PBPK tools in discovery stages to lead development and candidate selection increases efficiency, reduces the need for animal studies and increases PK understanding (Jones *et al.* 2009, Jones *et al.* 2012). PBPK studies can be used to prioritize preclinical compounds and select doses and formulation for *in vivo* animal PK and toxicological studies, whereas in candidate selection human pharmacokinetics and dose predictions are the key considerations (Jones *et al.* 2012). In the discovery phase, building a PBPK model for preclinical animals may also prove useful especially in the evaluation of mechanistic bioavailability issues after oral administration, or in the development of formulation and dose selection (Sinha *et al.* 2012, Bouzom *et al.* 2012). All the major commercial software applications for PBPK already contain at least two animal species in addition to human (Bouzom *et al.* 2012).

It is challenging to ensure good knowledge in understanding both the systemic 'virtual body' components and the drug-dependent ADME components

in building and refining PBPK models to be able to quantitatively understand the effects of intrinsic and extrinsic factors in PBPK simulation (Zhao *et al.* 2011, Bouzom *et al.* 2012). This will be further highlighted in the future when pharmacodynamic properties are linked to PBPK models to evaluate the dose-exposure-response relationship (Bouzom *et al.* 2012). Furthermore, as the PBPK understanding and respective models evolve and new clinical data become available from the earlier modeled compounds, there is a continuous need to re-simulate and re-evaluate the risks and benefits of existing (or even rejected) drugs (Rostami-Hodjegan 2012, Rostami-Hodjegan *et al.* 2012).

## 2.5 Metabolites in safety testing (MIST)

The awareness concerning drug metabolites' contribution to safety increased by the end of the millennium due to several reasons. First, analytical techniques had evolved to such a level that metabolites of drug candidates can easily be detected from human and animal samples. Secondly, *in vitro* methodologies to study metabolism had evolved as well, and together they facilitate better understanding of the role of metabolites (Baillie *et al.* 2002). The third reason was numerous drug withdrawals from the market based on idiosyncratic toxicities, especially hepatotoxicity. In the 1990s and 1980s, 14 drugs were withdrawn from the market in both periods due to hepatotoxic adverse events, whereas before 1980 the total number of hepatotoxicity related withdrawals was as low as 11 (Guengerich & MacDonald 2007, Smith & Obach 2005). Additionally, Smith & Obach (2006) reviewed 24 drugs withdrawn from the market in the EU and USA in 1980–2004 and found metabolites behind the presumed toxicity mechanism in 75% of the cases (14 confirmed and 4 suspected).

Earlier, metabolites were generally not considered, because their abundance and contribution to the pharmacological and toxicological profile of parent drugs was not known. However, in the early 2000s the time was right to study and consider their share in on-target and off-target activity, toxicological potential and safety in general. Concerning hepatotoxicity, something must have been done right, as in 2000–2013 there were only 7 new withdrawals due to hepatotoxicity (Guengerich & MacDonald 2007, Lexchin 2014, McNaughton *et al.* 2014). The data for 123 new drugs approved in Japan in 2000–2006 show that metabolite safety was studied in 39% of the cases (48 NMEs) and none of them produced metabolites having substantially greater toxicity compared to the parent compounds (Naito *et al.* 2007). The typical reason for the metabolite safety

testing was ‘It is the major metabolite’ (44/48); other important reasons were: ‘unique human metabolite’, ‘no metabolite in rat’ and ‘suspected genotoxicity’ (Naito *et al.* 2007).

### *2.5.1 The regulatory guidelines*

Publication of the so-called MIST paper (Metabolites In Safety Testing) in 2002 by the representatives of seven Big Pharma companies (Baillie *et al.* 2002) and the FDA response to that (Hasting *et al.* 2003, Baillie *et al.* 2003) started a scientific debate within the industry and the FDA concerning definition of major, minor and unique to human metabolites, their characterization and quantification and the need of toxicological testing of metabolites (Smith & Obach 2005, 2006, Davis-Bruno & Atrakchi 2006, Guengerich 2006, Humpreys & Unger 2006, Prueksaritanont 2006, Baillie 2007, Luffer-Atlas 2008). Ultimately this debate led to the final Guidance for Industry concerning safety testing of drug metabolites (U.S. Department of Health and Human Services 2008 (later FDA 2008)).

The guidance concentrates on human only metabolites and metabolites that are present at considerably higher levels in humans than in any of the animal species used in toxicology testing of the parent drug. In such a case, it is considered that the disproportionate metabolite may not have had adequate exposure during nonclinical testing and may need further examination due to potential toxicity, chemical reactivity or pharmacological activity. An active metabolite can enhance the therapeutic target activity or interact with other targets causing unwanted effects. However, if at least one animal species in toxicological testing has formed a disproportionate metabolite at approximately equal to or greater than human exposure level, it may be presumed that the contribution of that metabolite to the overall toxicity of parent drug has been taken into account (FDA 2008).

Phase I metabolites are more likely to be chemically reactive or pharmacologically active and, therefore, are more likely to need safety evaluation. Stable conjugates of the parent and phase II metabolites, including stable conjugates of potentially reactive intermediates, may eliminate the need for further safety evaluation. Metabolites raising a safety concern are formed at greater than 10% of parent drug systemic exposure at steady state measured in serum or plasma. Sometimes the disproportionate metabolites are not circulating, but only present in excreta. If a non-circulating disproportionate metabolite representing more than 10% of the bioavailable dose is found in urine or feces,

additional safety studies may be required. Such issues should be discussed with the FDA as soon as possible. The FDA encourages drug developers to study the metabolic profile of candidate drugs *in vitro*, *in vivo* (animals) and in humans as early as feasible to avoid finding disproportionate metabolites later and potential development and marketing delays (FDA 2008).

The primary approach to study toxicity contribution of a metabolite is to investigate the toxicity of parent drug in an additional animal species routinely used in toxicity studies forming the disproportionate metabolite at adequate exposure levels. The secondary approach is to synthesize the drug metabolite and study its toxicology. The recommended studies include general toxicity, genotoxicity, reproductive toxicity and carcinogenicity. The FDA acknowledges that direct dosing of a metabolite may lead to metabolism and toxicities that were not observed with the parent drug, adding complications to the safety evaluation (FDA 2008). The discrepancies include differences between the metabolite and parent drug in physico-chemical properties, interactions with transporters and metabolizing enzymes, tissue selective distribution and species differences in transporters and enzymes leading to potential downstream metabolite with unique toxicity (Prueksaritanont *et al.* 2006). The kinetics of synthetic metabolite and *in vivo* formed metabolite has been shown to be different, unless they both have a passive mechanism of membrane permeability and their enzymes for metabolism and removal are readily available in a well-mixed compartment (Pang 2009). Thus, similar behavior can only be expected from highly lipophilic parent drug and a metabolite that retains lipophilicity with minimal modification to the parent structure.

The decision to conduct direct safety testing of a metabolite is based on careful and comprehensive consideration of the following (FDA 2008):

- Similarity of the metabolite to the parent drug
- Pharmacological or chemical class
- Solubility and stability in stomach pH
- Phase I vs. phase II metabolite
- Relative amounts detected in humans vs. in animals
- Proposed drug indication and patient population
- The proposed duration of use and levels of exposure at the therapeutic dose

Additionally, the FDA emphasizes collection of all possible plasma samples from the nonclinical *in vivo* studies and first-in-human studies to be able to prove

adequate exposure in case of a disproportionate metabolite finding. For the disproportionate metabolite, FDA demands toxicological studies conducted under GLP and validated analytical methods capable of identifying and quantifying the metabolite (FDA 2008).

The European Medicine Agency revised its guidance on nonclinical safety studies (ICH guideline M3(R2)) for the conduct of human clinical trials at the end of 2009 to include short metabolite section (EMA 2009). The EMA requires nonclinical characterization for human metabolites having levels greater than 10% of total drug-related exposure and at greater levels than maximum observed in toxicological animal studies (EMA 2009). After guideline finalization a large number of questions arose concerning the metabolite section and they were formally answered in a Q&A addition to ICH guideline M3(R2) (European Medicine Agency (EMA) 2011). Q&A included definition and calculation method for 10% exposure; how to characterize safety, exposure and metabolites in different circumstances; and what kinds of studies are needed for metabolites (EMA 2011). The FDA has also unofficially adapted to these clarifications (Gao *et al.* 2013). It is noteworthy, from a regulatory perspective, that ICH guidance is prioritized over regional regulatory guidances.

### *2.5.2 The industry perspective*

There are remarkable costs associated with the demand to identify and quantify disproportionate metabolites. It means that the industry will not automatically study numerous metabolites in every animal study just in case the data is later needed. In 2006, the method development and validation for an additional metabolite incurred costs of about USD100,000, and the cost of analyzing the additional 10,000 samples from an extra preclinical program could end up at USD700,000 (Humpreys & Unger 2006). Moreover, the cost of the GLP toxicological program required would be (in 2009) between USD0.9 million and USD1.2 million, excluding the potential need for supporting studies (MTD, dose range finding, toxicokinetics). The cost for synthesis of kilogram quantities of the metabolite depends highly on the structure and requires at least an additional USD100,000 (Powley *et al.* 2009). Thus, the total cost would easily exceed USD2.0 million. Even if the cost alone were not a barrier to conducting safety evaluation of the metabolite, it is likely to lead to resource exchange from other development activities.

Smith & Obach (2009) performed a literature search for publications about quantitative assessment of human circulating metabolites and the same data for at least one animal species of the same parent drug. The search yielded 15 examples. Over half of the parents (eight) had two or more metabolites (average 3.5, range 0–13) present at level greater than 10% of the parent. In five of those eight cases the animal exposure was less than 10% of the parent for at least two metabolites (range 0–13) and in four of the earlier five cases one or two metabolites were not detected at all in animals (Smith and Obach 2009). Theoretically, if parent concentrations in circulation are very low, there can be an unlimited number of metabolites circulating at a level higher than 10% of the parent, thus requiring follow-up and resources of up to USD2.0 million each (Anderson *et al.* 2009). It has recently been proposed that the disproportionate metabolite qualification for additional studies should be agnostic to guidelines, indication, dosing and timing (Haglund *et al.* 2014). Individual industrial MIST case studies have been presented recently (Holmberg *et al.* 2014, Sharma *et al.* 2014, Sharma *et al.* 2015), as have tiered strategies concerning the MIST regulatory issues (Haglund *et al.* 2014, Ma & Chowdhury 2014).

Chemically reactive metabolites were practically left out from the MIST guidance for two reasons: they are generally short-lived and low in abundance (FDA 2008). Thus, they would not be detectable in the systemic circulation of animals or humans. It is also unclear how the information of reactive metabolites should be interpreted, as there still is a lack of understanding about the mechanisms of potential toxicity generated by metabolic activation (Baillie 2009).

## 2.6 Circulating metabolites

Primary human metabolites can quite easily be predicted *in vitro*, but it is more complex to understand which metabolites are likely to circulate and which are likely to be readily excreted or metabolized further.

### *2.6.1 Why metabolites circulate?*

Once a metabolite has been formed in the hepatocyte by metabolizing enzymes its fate is determined by its physicochemical nature. If the metabolite is lipophilic enough (positive LogP) and has a polar surface area less than 140–150 $Å^2$, it can passively diffuse to sinusoidal blood (and circulation) or bile. If a metabolite has a hydrophilic nature (LogP negative or slightly positive) and its PSA is higher than

75–100 Å$^2$, efflux transporters can carry the metabolite either to bile or sinusoidal blood (Smith & Dalvie 2012). Note the overlaps in both LogP and PSA; there are no definitive thresholds. Metabolites with higher polar surface area tend to be transported to the bile by canalicular efflux transporters (e.g. BRCP, P-gp, MRP2), whereas with lower PSA they tend to be carried to blood (and circulation) by sinusoidal efflux transporters (e.g. MRP3, MRP4, MRP6) (Benet 2010, Wu & Benet 2005, Giacomini *et al.* 2010). A worst-case scenario would be a metabolite being too hydrophilic to passively diffuse and too lipophilic to be actively transported. The resulting accumulation in hepatocyte could eventually cause a toxic effect. Also, extensive metabolism of a high-dose parent to a hydrophilic, high PSA metabolite could lead to rate limiting efflux clearance and concentration of the metabolite in the hepatocyte (Smith 2013). If the polar surface area of a metabolite with positive lipophilicity is below 75 Å$^2$, further metabolism is likely to happen (Smith 2011). Potential routes for metabolite disposition are depicted in Fig. 5.

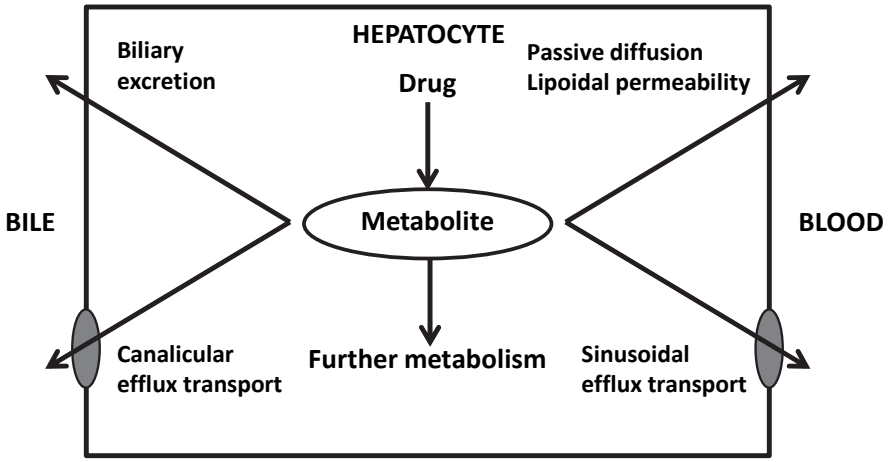


**Fig. 5. Potential routes for metabolite disposition from the liver (modified from Loi *et al.* 2013).**

Metabolites directed to bile will be eventually fecally excreted unless they are reabsorbed in the intestine either passively (lipophilic metabolites) or actively by uptake transporters. Glucuronide conjugates may also degrade back to parent drug or to phase I metabolite, and be either excreted or reabsorbed. Reabsorbed fraction will again face first-pass metabolism and the products will exit the liver either to bile or blood, actively or passively. Metabolites directed to circulation

may be excreted by kidney to urine, if they are hydrophilic enough to be taken up by the transporters at kidney proximal tubules. Nonexcreted (or effluxed) lipophilic metabolites will continue to circulate and distribute to tissues until finally excreted to bile or urine, or cleared by further metabolism and consecutive excretion.

Modern drugs are generally highly lipophilic with low PSA and they generate phase I metabolites with relatively small structural changes and evenly small changes in physicochemistry: usually a small decrease in LogP and a small increase in PSA due to hydroxylation. Such metabolites act very similarly to their parents, circulating, passively diffusing the membranes and primarily clearing via further metabolism (Smith & Dalvie 2012). However, a small change in physicochemical values makes the metabolites relatively stable compared to parent in terms of additional phase I metabolism. Depending on the place of hydroxylation (aliphatic vs. aromatic) enhanced phase II metabolism may occur. The pharmacological activity of a phase I metabolite may be retained, but usually with somewhat lower activity level than the parent has, as the parent has generally been designed for very high affinity and selectivity to a specific target (Smith and Dalvie 2012).

The physicochemical properties of a metabolite are changed dramatically when multiple steps of oxidative metabolism happen or the drug (or its primary oxidative metabolite) is conjugated with glucuronic acid or forms a sulfate. The lipophilicity of metabolite is considerably lower than that of the parent, and the PSA is much higher. As a consequence, membrane permeability drops significantly and active transport enzymes clear the metabolites mainly to bile (Smith & Dalvie 2012). However, many drugs have circulating glucuronides in high concentrations, even as major metabolites. It is likely that efflux transporter MRP3 at the basolateral membrane of the hepatocyte is responsible for the glucuronide transport to blood circulation (Zamek-Gliszczynski *et al.* 2006b, Hirouchi *et al.* 2009, Lagas *et al.* 2010). Sulfate conjugates have also been found in the circulation and there is evidence that the same transporter MRP3 (or MRP4) is involved (Zamek-Gliszczynski 2006b). Most circulating conjugative metabolites have low membrane permeability and thus do not distribute to tissues very well. The exception is zwitterionic glucuronides (or sulfates) from basic drugs that can retain some lipoidal permeability.

Glucuronides are usually highly protein bound (>50%) apart from acyl glucuronides, which are also rather unstable in plasma (Sawamura *et al.* 2010, Smith & Dalvie 2012), and the determination of their plasma protein binding

(PPB) needs careful experimentation (Buscher *et al.* 2014). However, the plasma stability of structurally closely related acyl glucuronides can be very different (Zhang *et al.* 2011). High protein binding of glucuronides is associated with the acid group in the glucuronide moiety. High protein binding of any drug or metabolite increases the duration of circulation due to decreased rate of clearance (Loi *et al.* 2013).

A study of plasma protein binding of 222 drugs reveals half of them having PPB higher than 90% (Zhang *et al.* 2012). As metabolites have generally lower lipophilicity than their parent has, their PPB is usually lower (Riccardi *et al.* 2015). Thus, major metabolites may have higher free fraction in circulation than their parents even if the circulating concentrations are the same (Smith *et al.* 2010).

### *2.6.2 Which metabolites circulate?*

Loi *et al.* (2013) conducted a literature survey of AUC values of 125 drugs and their main metabolites after human administration aiming to find out which kind of metabolites circulate with high metabolite-to-parent ratio (M/P ratio). They also calculated physicochemical properties (cLogD and tPSA) for the parent drugs and metabolites to monitor the trend changes within biotransformation classes (Loi *et al.* 2013). The results are summarized as follows:

#### *N-dealkylation*

- 42% of drugs formed primary or secondary amine metabolites (52/125)
- 77% of them had >25% M/P ratio (40/52)
- Average increase in tPSA was 20 Å
- Average decrease in cLogD was 1.0 log unit (cleavage ethyl/methyl unit)
- Four drugs had N-didesalkyl metabolites in circulation and 50% of them had >25% M/P ratio (2/4)
- Four drugs cleaved larger groups and 75% of them had >25% M/P ratio (3/4)

#### *Aliphatic/alicyclic oxidation or O-dealkylation*

- 32% of drugs formed aliphatic or alicyclic alcohol metabolites (40/125)
- 83% of them had >25% M/P ratio (33/40)

- 4% of drugs formed ketones to circulation (5/125) and 60% of them had >25% M/P ratio (3/5)
- Average increase in tPSA was 20 Å
- Decrease in cLogD was 1–2 log units
- Mostly remained as free aglycones due to high pKa (12–16) of the hydroxyl
- Primary alcohols were mainly further oxidized to carboxylic acids
- Aromatic/heterocyclic alkyl hydroxylations were further glucuronidated

*Arenol metabolites*

- 29% of drugs formed arenol metabolites (36/125)
- 50% of them had >25% M/P ratio for the remained aglycone (18/36)
- 22% circulated only as conjugates (8/36) and 75% of them had >25% M/P ratio (6/8)
- 41% circulated additionally as conjugates (15/36) and 47% of these conjugates had >25% M/P ratio (7/15)
- Low pKa (4–11) of the arenol group explains conjugation
- Increase in tPSA was 11–20 Å
- Decrease in cLogD was 0.5–1 log unit
- *para*-Hydroxylated arenols were mainly conjugated and *ortho*-hydroxylated remained as aglycones

*S-oxides*

- 10% of drugs formed S-oxide metabolites (12/125)
- 75% of sulfide drugs had sulfoxides >25% M/P ratio (6/8), but no/low sulfone metabolites in circulation
- Average increase in tPSA was 11 Å
- Decrease in cLogD was 1–2 log units
- 75% of sulfoxide drugs (3/4) had sulfone metabolites with >25% M/P ratio
- Conversion of sulfoxide to sulfone raise cLogD 1 log unit and tPSA 6 Å

*N-oxides*

- 9% of drugs formed aliphatic or aromatic N-oxide metabolites (11/125)

– 73% of them had >25% M/P ratio (8/11)
– Average increase in tPSA was 14 Å
– Average decrease in cLogD was 0.5–2 log unit

*Carboxylic acids*

– 9% of drugs formed carboxylic acid metabolites (11/125)
– 82% of them had >25% M/P ratio (9/11)
– Increase in tPSA was 20–40 Å
– Decrease in cLogD was 1.5–4 log unit

Additionally, seven drugs had rearrangement products as major metabolites, with 86% of them having >25% M/P ratio (6/7). Altogether, 88% of the drugs investigated had at least one metabolite with >25% M/P ratio and 96% had at least one metabolite with >10% M/P ratio. The data suggests the fraction of metabolite formed from the absorbed parent ($f_m$) being one of the major determinants for metabolite-to-parent ratio (M/P). It appears that $f_m$ value of >15% for a metabolite increases the likelihood to reach >25% M/P ratios especially in the case of amine, alcohol, S- and N-oxide and carboxylic acid metabolites (Loi *et al.* 2013).

### *2.6.3 In vitro prediction of circulating metabolites*

Before regulatory awakening to drug metabolites in safety testing, most of the reviews and comparisons of different *in vitro* test systems for metabolism concentrated on prediction of hepatic clearance, qualitative metabolic pathways or drug-drug interactions (Pelkonen *et al.* 2005, Pelkonen & Raunio 2005, Nassar *et al.* 2004, Brandon *et al.* 2003). These tasks are naturally highly important and the methods are well established. However, for the current safety interest of FDA and EMA – which metabolites circulate and give high *in vivo* exposure to humans – the prediction is still problematic and the results may not be accurate (Pelkonen *et al.* 2009a).

A study of 17 molecules from the Eli Lilly portfolio each having quantitative human *in vivo* and *in vitro* data (derived from varying *in vitro* systems) demonstrated that only 41% of the cases the *in vitro* data correctly predicted the *in vivo* circulating metabolites in human (Anderson *et al.* 2009). Underprediction occurred in 35% of the cases and overprediction in 24%. However, the prediction criteria were set strictly. The case prediction was successful when *in vitro*

metabolites were found at any level in human circulation. The data was considered underpredictive if a major circulating human metabolite was not identified in the *in vitro* system. One explanation might be the relatively high substrate concentrations used in standard metabolism screening, which may lead to saturation of metabolic enzymes. Some bias may have also been generated by using generic *in vitro* methods without optimization of the systems for each compound. Additionally, extrahepatic pathways are not covered in standard routines and some metabolites may have been missed due to that. In overpredictive cases, many *in vitro* metabolites were not seen circulating *in vivo*. Even if metabolites were observed in urine or feces, but not in plasma, the case was judged as overpredictive. In a typical case, *in vitro* system predicted too many CYP-mediated metabolites compared to *in vivo* circulation (Anderson *et al.* 2009). The more complex metabolism the parent generated the less likely it was that the *in vitro* methods predicted circulating metabolites. One explanation for this may be the analytical methods that were usually generic or optimized for the parent, as the instrumental response of a metabolite might be very different compared to the parent. Anderson *et al.* (2009) concluded that it is not possible to foresee which metabolites circulate in humans and what are the exposure levels. However, the *in vitro* and animal *in vivo* experimentations provide good knowledge of whether metabolism is likely to contribute extensively to drug clearance, and preliminary information on what metabolites might be the major circulating ones. Furthermore, it has been shown that metabolite to parent AUC ratio can be simulated from *in vitro* data reasonably well (Lutz & Isoherranen 2012). However, it requires thorough characterization of metabolite clearance pathways and understanding of the secondary metabolic processes involved in the elimination of the metabolite (Lutz & Isoherranen 2012).

In another industry study, Dalvie *et al.* (2009) from Pfizer incubated 27 in-house compounds and 21 commercial drugs with human liver microsomes, S9 fraction and hepatocytes to compare the *in vitro* metabolite profile to major human (*in vivo*) circulating and excretory metabolites. Only major *in vivo* metabolites were considered that constituted at least 10% of the circulating radioactivity or 10% of the radioactive dose in excreta. All in all, these 48 compounds produced 88 major circulating and excretory metabolites. Disappointingly, 31 of them (35%) were not detected in any of the *in vitro* systems. Hepatocytes predicted all major *in vivo* metabolites for 52% of the compounds, whereas S9 and microsomes were successful for 42% and 31%, respectively. The major differences were in the ability to produce secondary

metabolites by S9 and microsomes, and the lack of certain conjugation enzymes from microsomes. All systems had equal success to predict primary phase I metabolites (Dalvie *et al.* 2009). Fortunately, when examining only circulating *in vivo* metabolites produced by 37 of the study compounds, the success rates were much better. Hepatocytes predicted all major circulating metabolites for 65% of the compounds, whereas S9 and microsomes were successful for 62% and 49%, respectively. The differences were again in the production of secondary metabolites by S9 and microsomes, and the lack of ability of microsomes to produce all conjugations. All systems had equal success to predict primary phase I metabolites and S9 had best success to predict primary phase II metabolites (87% vs. 73% in hepatocytes and microsomes, respectively). Finally, prediction of primary metabolic pathways for 24 of the 27 Pfizer compounds that produced *in vivo* metabolites was extremely successful. Correct route was identified in 75%, 92% and 83% of the cases by hepatocytes, S9 and microsomes, respectively (Dalvie *et al.* 2009). However, for one compound none of the systems could predict the primary pathway that was direct glucuronidation of the parent. Even though the success rates to predict major primary metabolites and main metabolic pathways *in vitro* are high, the risk of finding a human *in vivo* disproportionate metabolite remains (Dalvie *et al.* 2009). However, despite the lack of absolute accuracy, the knowledge is rapidly accumulating and the data produced by current approaches is very useful for drug development (Pelkonen *et al.* 2009a).

Hepatocyte-like *in vitro* systems capable of producing quantitative amounts of drug metabolites in long-term incubations like HepaRG (Turpeinen *et al.* 2009, Anthérieu *et al.* 2010), micropatterened hepatocyte co-culture (Wang *et al.* 2010) or newly introduced dog hepatocyte co-culture (Atienzar *et al.* 2014), may prove to be useful in the hunt for *in vitro* systems to predict major human metabolites. Additionally, the systems can be used to produce large enough amounts of metabolites to be isolated and used as a reference standard for quantification. While HepaRG is likely to be utilized in toxicological studies, micropatterned hepatocyte is a really promising media for metabolite profiling of low clearance, slowly metabolizing drugs (Wang *et al.* 2010). Wang *et al.* (2010) used the same set of Pfizer compounds as Dalvie *et al.* (2009) with micropatterened hepatocyte co-culture and managed to detect 11 of the 31 undetected human metabolites in the previous study.

An alternative approach to metabolite prediction or isolation may be the use humanized chimeric mice (Sanoh & Ohta 2014, Katoh & Yokoi 2007, Bateman *et al.* 2014). Especially interesting would be continuous production (by excretion) of

even a small quantity of a disproportionate human metabolite that could be isolated and used as a standard. For the investigation of biliary excreted metabolites, the Entero-Test device has been successfully utilized in dogs to noninvasively capture biliary metabolites for qualitatively analysis (Guiney *et al.* 2010). As the device is routinely used also with humans in the clinic, it would be interesting to see its application for human metabolism study.

## 2.7 Qualitative and quantitative analysis of metabolites

The discovery phase bioanalysis of drugs and metabolites is a balancing act with speed and data quality of high number of samples, as the turnaround time of a bioanalysis sample set is only days (Ho 2014). In later drug development phases the game is more about data quality concerning the quantification of parent drug and important metabolites. To receive high data quality it is extremely important to know all the metabolites present in the samples and how they may impact the bioanalytical method and its selectivity (Garofolo *et al.* 2010). The bioanalytical methods need to evolve from drug discovery to drug development in terms of quality and method validation. The European Bioanalysis Forum has a best practice recommendation for a tiered approach in metabolite quantification (Timmerman *et al.* 2010). The first tier is 'Screening methods' for discovery bioanalysis without method validation and strict acceptance criteria. The second tier is 'Qualified methods' for preclinical investigations with scientific method validation implemented (e.g. accuracy, precision and stability). The final tier is 'Validated methods' for clinical bioanalysis which employs full method development and validation procedure according to Good Laboratory Practice (GLP). The tiers and drug development stages overlap depending on the project and anticipated results, e.g. a known toxic metabolite may trigger qualified methods for discovery bioanalysis and validated methods for preclinical bioanalysis (Timmerman *et al.* 2010).

### *2.7.1 Detection and identification of drug metabolites*

The recent development of both high resolution liquid chromatography (e.g. UHPLC, UPLC) and high resolution mass spectrometry (e.g. TOFMS, QTOF, Orbitrap) has made metabolite detection an almost trivial task (Ravindran *et al.* 2014, Tolonen & Pelkonen 2015, Zhu *et al.* 2011, Ma & Chowdhury 2011, Tolonen *et al.* 2009, Prakash *et al.* 2007). Recent comparison of different mass

spectrometric instruments used in metabolite profiling was compiled by Tolonen & Pelkonen (2015). These workhorses of any discovery DMPK laboratory with qualified analytical scientists can easily be utilized to detect potentially all predicted and unpredicted *in vitro* metabolites with a reasonable dynamic range for parent depletion, good sensitivity to detect also minor metabolites and excellent mass resolution and mass accuracy to determine exact molecular mass and elemental composition for the found metabolites. The instrument software automatically calculates metabolic stability of parent, relative abundances of all found metabolites and proposes their biotransformation to help medicinal chemists evaluate clearance rate, metabolic soft spots and initial metabolism pathways. The post-acquisition data mining for metabolite detection utilizes comparison to control sample (Mortishire-Smith *et al.* 2005), background subtraction (Zhu P. *et al.* 2009a, Zhang & Yang 2008), predicted ion extraction (Chang *et al.* 2000, Castro-Perez *et al.* 2005), mass defect filtering (Zhu *et al.* 2006, Zhang *et al.* 2008, Zhang *et al.* 2003) and isotope pattern filtering (Cuyckens *et al.* 2009, Zhu P. *et al.* 2009b). Automation of such systems enables first-line metabolite profiling of tens of new chemical entities in a day from different therapeutic programs and diverse structural classes (Tiller *et al.* 2008a).

The structural characterization and complete identification of metabolite structure requires a bit more from the MS instrument and software. Besides the above-mentioned instruments the modern drug metabolism laboratory has additional instruments for metabolite identification and characterization (e.g. MS/MS, ion trap, linear trap, FTMS). The instruments are able to selectively (MS/MS or $MS^n$ ability) or non-selectively (in-source fragmentation or $MS^E$) fragment the metabolite ions and generate structural information. Software can be used to switch the MS to operate in a certain data acquisition mode triggered by the data gained from full scan mode. While full scan mode acquires all ions with preset parameters, triggered data acquisition mode can, for example, scan and acquire every second scan differently. Additionally, the full scan operation can have preset alternating fragmentation energies (high and low collision energy every second scan, $MS^E$ scan) enabling simultaneous acquisition of molecular ions and structure specific fragments (Mortishire-Smith *et al.* 2005, Wrona *et al.* 2005). Possible data acquisition modes include triggering based on ion intensity above preset threshold (Ruan *et al.* 2008), detection of certain neutral loss (Bateman *et al.* 2009), detection of isotope pattern (Cuyckens *et al.* 2008, Lim *et al.* 2008, Rathahao-Paris *et al.* 2014) or detection of specific mass defect (Bateman *et al.* 2007, Tiller *et al.* 2008a). In addition, the earlier mentioned post-

acquisition data mining methods product ion filtering (Ruan *et al.* 2008, Lim *et al.* 2007, Wrona *et al.* 2005) and neutral loss filtering (Ruan *et al.* 2008, Wrona *et al.* 2005) can be used. Automated systems containing a metabolite database may permit full characterization of major metabolites from *in vitro* and *in vivo* samples, decreasing the workload of metabolism scientists (Bonn *et al.* 2010, Li *et al.* 2009). In cases where fragmentation does not give enough information to distinguish between monoisotopic metabolites (e.g. hydroxylation vs. S/N oxidation) hydrogen/deuterium exchange can be a useful technique for molecules containing limited number of exchangeable hydrogens (Liu *et al.* 2007, Tolonen *et al.* 2005). For compounds having numerous exchangeable hydrogens, such as glucuronide conjugates, ion mobility spectrometry combined with mass spectrometry (IMS-MS) instrument can be a wonderful structural elucidation tool even for co-eluting conjugates (Shimizu *et al.* 2012) or co-eluting parent and glucuronide (Smith *et al.* 2013) from very complex *in vivo* matrices (Blech & Laux 2013).

Even though very confirmatory metabolite structures can be obtained, MS data alone does not prove the structure definitively, but isolation and NMR characterization, or synthesis of major metabolites and direct comparison of *in vivo* or *in vitro* metabolites is needed (Frederick & Obach 2010, Walker *et al.* 2014). Typical analytical process for detection and identification of drug metabolites has been presented by Ma & Chowdhury (2011).

### *2.7.2 Quantification of metabolite exposure without standards*

Quantification of the metabolite levels from different *in vitro* systems and *in vivo* matrices is required to be able to evaluate human exposure. The above-mentioned LCMS data cannot be used for quantitative comparisons between metabolites and parents, because ionization efficiencies and mass spectrometric responses typically differ (Frederick & Obach 2010). A huge metabolite response in LC/MS does not necessary mean that the metabolite has major presence, but the abundance data is very tentative. The response differences are due to different physicochemistry of the metabolite and physicochemical properties affect ionization directly. Furthermore, metabolites are usually more polar than the parent and elute out from the HPLC earlier than the parent. Earlier elution time means more water in the HPLC mobile phase, which affects negligibly the ionization efficiency in the MS interface. Additionally, the matrix effect will be different if the elution time is different. Dahal *et al.* (2011) tested the response

difference with a set of very similar substrates and their common metabolites containing multiple nitrogen atoms to guarantee good LC/MS signal. None of the studied biotransformations, O/N-demethylations and aromatic/benzylic hydroxylations, gave a consistent trend of bigger or smaller signal compared to the parent. The study demonstrates that 90% of the parent-metabolite responses were statistically different and the ratio of the slopes of standard curves varied up to 4-fold even though the main structure remained very ionization friendly (Dalal *et al.* 2011).

The use of LC/UV response correction for LC/MS peak areas has been proposed to obtain quantitative information of metabolites without authentic standards (Vishwanathan *et al.* 2009). Two important prerequisites are that the UV spectrum of the metabolite is very similar to the parent and the metabolite peaks in the LC/UV chromatogram are resolved without any interference. Unfortunately, the latter is not obvious with *in vivo* samples without extensive workout and potential loss (and chemical modification) of the metabolites. The UV correction method was tested against actual calibration with one compound producing six metabolites to rat plasma after oral administration (Yang *et al.* 2011). The results show up to three-fold differences in $C_{max}$ and AUC, which is actually quite reasonable for early decision-making. Yang *et al.* (2011) also studied metabolite calibration against MS calibration curve of the parent and found up to eight-fold differences compared to actual calibration with metabolite standards. However, both of the methods (UV correction and parent MS calibration) demonstrated only about two-fold difference for the four most parent-like metabolites (N-oxide and aromatic/alicyclic hydroxylations). This sounds good, but unfortunately, the metabolite with eight-fold difference (parent MS method) was by far the most important metabolite whose PK would have been significantly underestimated (Yang *et al.* 2011).

Radiochromatographic metabolite profiles can be used to create relatively accurate MS response factors for abundant metabolites, since the specific activity of the radiolabeled parent is known. However, the synthesis of radiolabeled analogs usually takes place only for serious lead molecules quite late in the preclinical phase or just before the initiation of clinical studies. The method is simple (Yu *et al.* 2007):

1. Analysis of the same metabolized sample with the same chromatographic conditions by LC/MS and LC/RAD.
2. Obtain definitive metabolite concentrations from the radiochromatogram.

3. Comparison to respective LC/MS peak areas.
4. Calculation of response ratios for each metabolite.
5. Use the response ratios to quantitate metabolites from unknown *in vitro* or *in vivo* samples.

Matrix mixing of reference sample before response ratio measurement ensures minimal matrix effect difference to the unknown samples, and the use of long LC method for unknown samples helps bridging metabolites unambiguously. The caveats are rather poor sensitivity of LC/RAD (minor metabolites may be missed or response ratio is less accurate) and unknown linearity due to only one-point calibration. Adjusting reference sample to the same concentration level than expected unknown average concentrations helps somewhat with the linearity issue. The method can provide quantitative results within 20% compared to concentrations determined from the radiochromatogram (Yu *et al.* 2007). The method is in routine use within the pharmaceutical industry (Tiller *et al.* 2008b).

NMR is an attractive technique for quantitative determinations as the signal response is independent of the molecular structure of the analyte. In principle, known molar response from any $^{1}H$ resonance of any compound can act as a reference standard for any $^{1}H$ resonance of any other compound. It is even possible to use a mathematically generated signal inserted into the spectrum as quantitative reference (Walker *et al.* 2011). The limitations of the technique are high price (and thus availability), low sensitivity (better with expensive cryoprobes) and interfacing with chromatographic systems (not common, high price, low sensitivity due to void volumes, need to stop flow during metabolite to enhance sensitivity). In practice, NMR quantification of metabolites has been done for isolated metabolites from various biological matrices (Gear *et al.* 2008, Espina *et al.* 2009, Srivastava *et al.* 2010, de Vlieger at al. 2010, Mutlib *et al.* 2011), which provides good sensitivity down to 10 ng/mL (Gear *et al.* 2008) and generally good accuracy producing concentrations within 10% of the nominal value (Espina *et al.* 2009).

The regulatory guidances do not actually require determinations of steady state concentrations of metabolites in animals and humans; it is only necessary to demonstrate animals having greater exposure than humans (FDA 2008, EMA 2009). In fact, characterization of metabolite toxicity is considered adequate even when animal exposure is 50% of that in human (EMA 2011). As a consequence, a simple LC/MS/MS method with pooled samples can provide quantitative comparisons of exposures to all detectable metabolites between animal species

and humans without synthetic metabolite standards and calibration curves as proposed by Walker *et al.* (2009) and confirmed by Ma *et al.* (2010). Gao *et al.* (2010) additionally demonstrated that MS exposure ratios correlated extremely well (slope 0.99, r = 0.994) with the nominal concentration ratios. Furthermore, it was shown that the actual exposure ratio was at least 1.0 if the MS exposure ratio was at least 2.0 ($p < 0.01$) (Gao *et al.* 2010).

Human steady state pharmacokinetic samples and animal toxicokinetic samples are pooled using Hamilton plasma pooling technique (Hamilton *et al.* 1981, Hop *et al.* 1998). If required, new animal samples should be generated at steady state and NOAEL dose level. Pooled samples are mixed with control matrix to minimize LC/MS matrix effect, and internal standard is used. After LC/MS/MS analysis the exposure multiples (EM) are calculated using the equation (1)

$$EM = (A^{m,a}/A^{IS,a})/(A^{m,h}/A^{IS,h}) \quad (1)$$

where $A^{m,a}$ and $A^{m,h}$ are the metabolite peak areas measured from animal and human samples, respectively, and $A^{IS,a}$ and $A^{IS,h}$ are the peak areas of internal standard measured from animal and human samples, respectively. Safety margin of 0.3 (EM > 1.3) is proposed based on doubling the accuracy demand of standard bioanalytical method validation (Ma & Chowdhury 2011). As a conclusion from the method, if the exposure ratio is greater than 1.3 for a metabolite, it should be investigated whether this metabolite is >10% of total drug-related material in humans (Ma & Chowdhury 2011). The method was proved to be applicable also for potentially unstable (during storage) epoxide, amide catechol and acyl glucuronides metabolites, but not for iminium or ester metabolites (Gao & Obach 2012). This approach has been unofficially verified by the FDA to be adequate for the determination of metabolite exposure (Gao *et al.* 2013). The method offers time- and resource-savings in determining metabolite exposures in humans offering an opportunity to postpone synthesis of radiolabeled compound for mass balance studies. It should be kept in mind although that this approach does not remove the risk of later detecting a disproportionate metabolite in humans, which still requires definitive ADME studies with radiolabeled compound in humans and animals.

### 2.7.3 ADME study strategies in humans and animals

After the MIST guidance release (FDA 2008) a number of proposals for comprehensive strategies of human and animal metabolite identification and human exposure have been published by the pharmaceutical industry (Nedderman 2009, Nedderman *et al.* 2011, Leclercq *et al.* 2009, Zhu M. *et al.* 2009, Penner *et al.* 2009, Penner at al. 2012, Isin *et al.* 2012, Obach *et al.* 2012) using various analytical methodologies including definitive human and animal ADME studies with radiolabeled compound. These strategy papers contain discussion about the need and timing of individual *in vitro* and *in vivo* ADME studies with humans and animal species, proposals for accelerating or delaying human radiolabeled studies, and workflows or decision trees for circulating metabolite characterization and exposure determination. The most radical thinking by Pfizer scientists proposes the complete removal of routine animal radiolabeled ADME package from drug development and continuing with human-only radiolabeled ADME approach (Obach *et al.* 2012, Obach *et al.* 2013). They feel that the removal could be done because of following reasons:

- The MIST guidance clarifies that circulating human metabolites are the most important ones.
- The exposures of human metabolites in preclinical toxicity species are more important than complete metabolite profile in animal species.
- The analytical technologies are advanced enough to facilitate identification and characterization of very low-level metabolites from humans and animals from unlabelled compounds.

In this strategy, a radiolabeled drug is first used in a rat WBA study to get tissue dosimetry data before a human radiolabel study. In the human radiolabeled study, the metabolites comprising 10% of the total circulating drug-related material are detected from the quantitative metabolite profiles. For those metabolites, relative steady-state metabolite exposures across species are determined using MS metabolite ratios as described earlier (Ma & Chowdhury 2011). Simulated steady state AUC and $C_{max}$ values can be obtained from a single radiolabeled dose, if unlabeled steady-state data is available (Prakash *et al.* 2012). Finally, actions are taken according to human vs. animal exposure multiples. Downstream metabolites of reactive metabolites found from the excreta are compared similarly (Obach *et al.* 2012). Radiolabeled ADME animal studies should still be conducted

as problem-solving studies when certain risks need to be assessed (Obach *et al.* 2012):

1. Addressing specific animal toxicity and search for animal-unique metabolite or disproportionate metabolite relative to human
2. Assessing potential for biliary excretion with bile duct cannulated rats
3. Confirming (poor) oral absorption
4. Addressing low mass-balance in humans to find out if material is retained in the body
5. Finding macromolecular plasma protein adducts
6. Finding reactive metabolites as downstream products of reactive intermediates
7. Understanding clearance pathways.

The proposal was agreed by AstraZeneca scientists with the exception to propose retaining routine radiolabeled QWBA, mass balance and ADME studies with one animal species, namely rat, to better understand the complete metabolism picture of the drug candidate (Isin *et al.* 2012). The propositions provoked an industry-wide commentary emphasizing the general benefits of the traditional radiolabeled animal study package in interpreting preclinical toxicological and pharmacological findings, characterizing circulating metabolites and determining principal clearance pathways (Penner *et al.* 2012, White *et al.* 2013). They find radiolabeled animal ADME studies an irreplaceable component of drug development that need to be performed before exposure of human subjects to investigational new drugs.

The general differences between the primary objectives of human and animal radiolabeled ADME studies are small, as can be seen from Table 3 (Penner *et al.* 2009, Penner *et al.* 2012). It is naturally clear that dosing regimen, number of doses and sample collection suffer great limitations with humans compared to animals. With animals the options include multiple high doses and radioactivities with several administration routes, bile duct cannulation, terminal blood collection and even organ removal. With humans, one is typically limited to one oral dose (Phase II clinical trial dose level) of radiolabeled compound (100–200 μCi activity) to male volunteers and collection of primarily three types of samples: blood, urine and feces. The human sample collection usually continues until about 90% of administered radioactivity is excreted or only 1% of radioactivity is excreted in two consecutive days. However, this may take up to ten days of the study subject's time (Penner *et al.* 2009). Generally, the recovery

of radiolabeled material is at least 80% in humans, whereas in rat and dog it is somewhat greater, at least 90% and 85%, respectively (Roffey *et al.* 2007). Possible reasons for low mass balances include non-covalent tissue sequestration, affinity to phospholipids, binding to melanin or to specific proteins in tissues and covalent binding. However, the low mass balance results do not automatically implicate safety issues, as the usual reasons involve long half-life of the drug candidate or its metabolites (Roffey *et al.* 2007). As it is not possible to thoroughly characterize the distribution of drugs and metabolites to human tissues and and organs, the human ADME studies are actually human AME studies. For simplicity, the acronym ADME is used for both animals and human.

**Table 3. The primary objectives of human ADME/MB studies and animal ADME/MB studies (collected from Penner *et al.* 2009 and Penner *et al.* 2012).**

| Primary objective | Human ADME | Animal ADME |
|---|---|---|
| To determine mass balance | x | x |
| To determine routes of elimination | x | x |
| To determine extent of absorption | - | x |
| To identify circulatory and excretory metabolites | x | x |
| To determine clearance mechanisms | x | x |
| To characterize the distribution into tissues and organs | - | x |
| To determine exposures of the compound and its metabolites | x | x |
| To help validate animal species for toxicological tests | x | x |
| To explore if metabolites contribute to pharmacological or toxicological effects | x | x |

Secondary objectives of human ADME studies may include the evaluation of DDI potential and identification of disproportionate metabolites (Penner *et al.* 2009). Despite the dosing and sample collection limitations, a radiolabeled human ADME study is the golden standard study in drug development to understand the metabolic properties of a drug candidate in humans (Penner *et al.* 2012). It gives a definitive quantitative answer as to clearance through circulating and excretory metabolite profiles, triggers additional studies to address potential metabolic safety liabilities, and helps to understand the impact of metabolism on drug efficacy.

It is possible to summarize different ADME study approaches of the pharmaceutical industry into four basic strategies based on the nature and timing of principal human and animal radiolabeled ADME studies (Nedderman *et al.*

2011). These basic strategies are the 1) standard, 2) accelerated, 3) delayed and 4) early ADME approaches and they are shown in the first four columns of Figure 6.

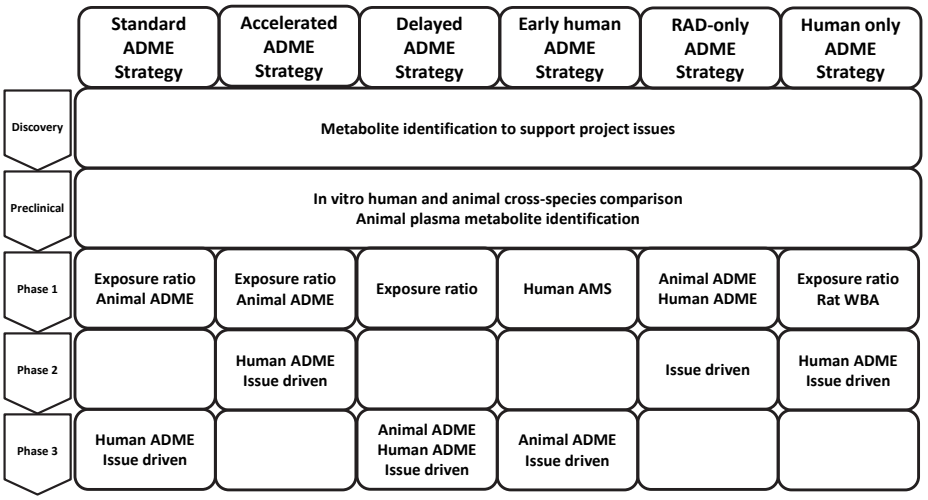


**Fig. 6. A schematic presentation of basic ADME strategies used by the pharmaceutical industry according to Nedderman *et al.* (2011), Penner *et al.* (2012) and Obach *et al.* (2012).**

The standard approach has for years been the most common strategy in the pharmaceutical industry. Usually, radiolabeled animal studies are conducted simultaneously to Phase I clinical stage to gain human versus animal exposure comparison (Exposure ratio). Typically, quite extensive radiolabeled animal ADME studies are conducted with two toxicology species, a rodent and a nonrodent, rat and dog being the most commonly used species. Definitive human ADME study with radiolabeled compound is delayed to very late in development, just before entering Phase III clinical studies, together with additional and issue-driven animal studies. The standard approach delays the burden of costs associated with definitive human ADME study and also delays the exposure of humans to the radiolabeled compound. However, the approach bears risks of wasting time with animal-specific issues and the appearance of unpleasant human-specific metabolism surprises at a later stage (Nedderman *et al.* 2011).

The accelerated approach is usually not preselected unless very short late development timelines are expected (e.g. oncology indication). Generally, early human definitive studies right after Phase I are triggered by scientific considerations that emerge in Phase I and animal radiolabeled studies. This

approach has high early cost burden, but the results may accelerate later development (Nedderman *et al.* 2011).

In the delayed ADME strategy, all the radiolabeled studies (human and animal) are postponed until the late Phase II clinical stage to save resources. This is a risky approach, as the Phase II stages are full of development activities; this raises the possibility of a costly delay in starting the Phase III program if serious metabolism issues emerge (Nedderman *et al.* 2011).

The early human approach utilizes accelerator mass spectrometry (AMS) technology and very low radioactive dosing, which enables early human administration, simultaneously with or even before Phase I, without the need of a rat tissue distribution study (WBA, Zhu M. *et al.* 2009). In the best case, the human information gained is definitive, no further human radiolabeled study is needed, and despite the high cost of the study an ultimate cost-efficiency of 50% may be achieved (Yamane *et al.* 2013). However, the risk of wasting money may occur due to possible early discontinuation of the drug candidate or nondefinitive AMS data (Nedderman *et al.* 2011).

In another early human strategy recently proposed, animal radiolabeled ADME studies are conducted at the beginning of Phase I and human definitive ADME study immediately after that, before ending Phase I (Penner *et al.* 2012). The radical difference to the other strategies is the complete lack of human-animal exposure studies with unlabeled material. One could call this radiolabeled only ADME strategy (fifth column, RAD-only strategy in Fig. 6). The human only ADME strategy (Obach *et al.* 2012) would be the sixth approach (Fig. 6), where only a rat WBA study would be conducted simultaneously with the Phase I clinical study and human-animal exposure comparisons. Definitive human ADME study would be conducted in early Phase II and issue-driven animal studies (radiolabeled or not) after that, before the initiation of clinical Phase III studies.

Radiolabeled material is not only used in human and animal definitive ADME studies during clinical development; they are also widely used in preclinical and exploratory phases as well, e.g. to increase detection lead molecules in biological assays or gain quantitative *in vitro* metabolism data (Isin *et al.* 2012). At earlier stages $^{3}H$ label is preferred due to relative ease of synthesis, but there is a risk of losing the label in case of oxidative metabolism and inducing kinetic isotope effects to certain biotransformations. The preparation of $^{14}C$ label is much more expensive as the label has to be introduced to the core of the molecule skeleton early in the synthetic pathway. This ensures the protection of the label against metabolism, but due to the cost of processing the

chemistry $^{14}C$ label is generally used for serious drug candidates only (Isin *et al.* 2012).

### *2.7.4 Microdosing*

The concept of microdosing was introduced to get drug candidates into humans faster and to obtain pharmacokinetic and metabolism data of one or several drug candidates prior to the actual first-in-human (FIH) study and Phase I (Garner 2005, Brown *et al.* 2006). The concept is also called Phase 0. The regulatory guidances (FDA 2006, EMA 2009) allow clinical study with a microdose of 100 µg and <1% of the expected pharmacological dose of drug substance. The substance can be isotopically labeled or not. Even repeated allowable doses (up to 5 doses) are possible (EMA 2009). The preclinical testing program for a microdosing trial with a sub-pharmacologic dose can be less extensive than a traditional test package before FIH as the potential risk for human subject is much lower (FDA 2006). The regulatory requirements for safety testing before a microdosing study have been comprehensively collected by Muller (2011). Due to the low dose, no efficacy or safety data can be expected from a microdosing study.

Initially, the concept was introduced to AMS analytical technology due to superior sensitivity compared to other techniques, but in recent years the sensitivity of LC/MS/MS technologies has developed to such levels that microdosing and even cocktail microdosing studies of favorable candidates can be conducted and analyzed without isotopic labeling (Maeda and Sugiyama 2011). In AMS studies, the natural $^{14}C$ content in human tissues limits the detection level to pM region, but experimentally high attomolar levels have been reached with a sensitive carbon carrier method (Salepour *et al.* 2009). The benefits of microdosing include about 6–12 months faster FIH results than traditionally, with at least USD1 million less in expenses and need of only sub-milligram quantities of material (synthesis under limited GLP) compared to about 100 grams needed (synthesis under strict GMP) until Phase I completed (Shinde *et al.* 2011). The drawbacks are the high price of the AMS instrument, demand for highly skilled operators, very laborious sample preparation (graphitization) and lack of automation. The AMS instrument cannot be directly interfaced with HPLC, and currently, metabolites need to be fraction-collected before analysis. Instrument size, price and cost of use have recently come down somewhat without compromising the quality of results, but they are still big-room-size instruments

demanding about 60 $m^2$ floor area (Young *et al.* 2008). There have also been advancements in building a direct analysis interface for nonvolatile samples to avoid graphitization for faster sample preparation (Liberman *et al.* 2004). It is anticipated that the investment to AMS instruments within the pharmaceutical industry will increase as new generation instruments become commercially available (Young and Ellis 2007). However, the real breakthrough would be hyphenation with HPLC.

There has been a suspicion that the lack of enzyme and/or transporter saturation that may happen with the therapeutic doses of drugs affects the PK and metabolism profile of a drug with microdosing. Pharmacokinetic comparison between microdose and therapeutic dose of many drugs shows the results to be very well comparable and the data would have been useful in the selection or de-selection of drug candidates (Lappin *et al.* 2006, Lappin *et al.* 2011, Maeda and Sugiyama 2011). As modern drugs tend to have greater potency and lower doses than in the past, the enzyme and transporter saturation issue might become irrelevant and a microdose could be expected to predict the therapeutic dose pharmacokinetics quite well (Lappin *et al.* 2011). Furthermore, PBPK analysis based on microdosing results and *in vitro* studies on metabolism, transporters, binding and DDI enables accurate prediction of PK profiles in humans after the therapeutic dose (Sugiyama and Yamashita 2011). However, microdosing can only allow assessment of PK properties, but the common causes of attrition lie in safety and efficacy. Thus, microdosing may lead to savings on some of the overall expenses, but would likely have limited impact on the overall productivity of drug development (Boyd and Lalonde 2007).

### *2.7.5 Microtracing*

Simultaneous dosing of a clinical dose (oral, dermal, sublingual etc.) of nonlabeled drug and an i.v. microdose of isotopically labeled drug is called microtracing. The aim is to determine human clearance, volume of distribution, total metabolism and excretion profiles and absolute bioavailability of a drug (Dueker *et al.* 2011). As the total exposure is at therapeutic level, it is not microdosing. The microdose of labeled compound is at a similar low level as in microdosing, so a rat WBA study is not needed, but for the therapeutic dose of nonlabeled compound all the prerequisite regulatory requirements have to be fulfilled. Typically, this kind of single exposure absolute bioavailability study is conducted together with Phase I clinical studies (Obach *et al.* 2012). The labeled

drug and metabolites are analyzed by preparative HPLC and AMS whereas nonlabeled drug and metabolites are analyzed with traditional LC/MS and MSMS methods. With careful sample preparation and analytical operation the AMS approach meets the bioanalytical guidance requirements similar to those of LC/MS/MS assays (Gao *et al.* 2011).

Simultaneous intravenous pharmacokinetics and oral (or other) route pharmacokinetics together with other *in vivo* and *in vitro* data and PBPK modeling allow the determination of absolute bioavailability, mass balance, metabolite exposure and other key PK parameters (Zhu M. *et al.* 2009, Dueker *et al.* 2011). In the best case, conventional human ADME (labeled or nonlabeled) are not needed (Zhu M. *et al.* 2009). The microtracing study design has six advantages over traditional approaches (Dueker at al. 2011):

- The microdose formulation is simplified and the microdose is too small to perturb the kinetics of the therapeutic dose.
- Animal studies enabling intravenous dose are not needed (e.g. tissue dosimetry and local tolerance studies).
- The two doses are mixed *in vivo* ensuring equal clearance.
- The determination of clearance, routes of excretion and second-pass metabolism is enabled by $^{14}C$ microdose.
- Statistical power is improved because individuals serve as their own control.
- The very cost-efficient single arm study design delivers the same benefits as traditional two tests with a cross-over design with a washout period.

The microdose is so low that excreted radioactivity contamination is not a concern. Additional cost-efficiency is reached when the need to house the study subjects in the clinic for the whole sample collection period is removed (Obach *et al.* 2012). The microtracing approach allows different multiple dose study designs, e.g. to measure exact steady state metabolite concentrations or to compare metabolism profiles between the first and last day of administration to address auto-induction or inhibition (Obach *et al.* 2012). With high-sensitivity AMS, the reportable plasma concentrations can be routinely measured until 48 h post-dose, which results in better overall PK profiles (Gao *et al.* 2011). Microtracing has been successfully used to study long half-life compounds or poor absorption, both of which require very long collection periods with low sample concentration of drug candidate and metabolites (Graham *et al.* 2011).

Microtracing can also be used for compounds suffering from radiolytic instability in conventional human $^{14}C$ ADME studies (Comezoglu *et al.* 2009).

## 2.8 Metabolism-related adverse drug reactions

Adverse drug reactions (ADR) are important safety reasons for attrition during drug development, and to a great extent they are generated by stable or reactive metabolites. A key issue in reducing the drug development cost and time is to detect and assess metabolism-related toxicities as early as possible. Simple *in vitro* testing in the discovery phase may save up to hundred million euros when preventing a toxic drug from entering clinical trials (Guengerich & MacDonald 2008).

ADR toxicity has been categorized into five classes based on their clinical, chemical and pharmacological characteristics (Park *et al.* 1998). These toxicity classes are Type A: augmented reactions generated by circulating metabolites, further sub-classified to A1 (on-target pharmacology) and A2 (off-target pharmacology) by Smith & Obach (2005); Type B: bizarre or idiosyncratic drug reactions (IDR) caused primarily by reactive metabolites and often mediated by the immune system; Type C: chemical reactions caused directly by reactive metabolites; Type D: delayed genotoxicity or teratogenicity reactions arising months or years after the exposure to reactive metabolites; and Type E: end-of-treatment reactions related to the pharmacology of the drug (similar to Type A), a sudden stop of the medication (not return of disease), and possibly involving circulating metabolites (Park *et al.* 1998, Frederich & Obach 2010). Type A, B, C and D toxicities and metabolism are reviewed more thoroughly.

### *2.8.1 Type A1 toxicity*

For type A1 toxicity (target activity), the administered drug is often behind the most common exaggerated pharmacological effects as the parent compound is designed to be the most active component present (Park *et al.* 1998, Smith & Obach 2005, Smith *et al.* 2009). The effects are usually predictable from the known pharmacology, dose-dependent and occur due to too high receptor occupancy or enzyme inhibition, or due to a target response in nontarget tissue (Park *et al.* 1998, Testa 2009). The causative factors include poor dose selection (overdose) and individual variability due to metabolic or transporter polymorphism (Smith & Obach 2009). Circulating stable metabolites having

minor structural modifications to noncritical substituents in terms of activity and physicochemisty compared to the parent can also produce Type A1 toxicity as augmented pharmacological effect to the target. Particularly, unbound concentrations of such active metabolites are available to the intended receptors causing the extra effect (Smith & Obach 2009). Metabolites that exceed 25% of the parent in free concentration should be considered for the study of their on-target activity (Smith &Obach 2005, Smith &Obach 2009). The onset of the effect is usually almost instant (Smith & Obach 2009) and due to the high dose-dependency the symptoms may be alleviated by dose reduction (Park *et al.* 1998).

### *2.8.2 Type A2 toxicity*

For type A2 toxicity (secondary pharmacology, off-target activity), the administered drug is again most often behind this selectivity issue (Park *et al.* 1998, Smith & Obach 2005, Smith *et al.* 2009). The effects are generally dose-dependent, predictable from the known selectivity and pharmacology of the parent drug, and occur due to interaction with a nonintended target (Park *et al.* 1998, Testa 2009). Circulating stable metabolites having minor structural modifications to noncritical substituents in terms of activity and physicochemisty compared to the parent can produce Type A2 toxicity similar to the parent (Smith & Obach 2009). Major metabolic modifications can lead to surprising secondary pharmacological effects (novel compared to the parent), but this is a very rare event (Smith & Obach 2005). Again, unbound concentrations of such active metabolites are available to the nonintended receptors causing the unwanted effect (Smith & Obach 2009). It has been recommended to study off-target selectivity if a free concentration of a circulating metabolite exceeds 1 μM, regardless of the structure (Smith & Obach 2005, 2006). The onset of the effect is usually almost instant, but can take up to one month, if a tissue change is needed for the expression of toxicity (Smith & Obach 2009, Smith *et al.* 2009).

### *2.8.3 Type B toxicity*

Type B (bizarre) toxicity is usually referred to as idiosyncratic drug reaction (IDR) due to its unpredictable nature that is not related to the pharmacology of the drug. The IDRs are very rare (<1 case in 5,000 patients) and the mechanism does apparently not follow a simple dose-response relationship in a classic manner even though drugs given at high doses are more likely to cause these reactions

(Park *et al.* 1998, Uetrecth 2007, Testa 2009). The mechanisms are poorly understood, might contain genetic and external factors and are generally considered patient-dependent. Additionally, the mechanisms of IDRs seem to have a metabolic and/or immunologic component (Park *et al.* 1998, Uetrecht 2007, Testa 2009, Frederich & Obach 2010). These potentially life-threatening hypersensitivity reactions include anaphylaxis, hepatoxicity, aplastic anemia and drug-induced allergy. The three primary sites of type B toxicity are liver, blood cells and skin, possibly due to their high content of oxidizing systems and the presence of active immunological defense. Many of the IDR victims suffer toxicity effects in all three sites (Park *et al.* 2000, Uetrecht 2003, Smith *et al.* 2009). Type B toxicity has a fairly rapid onset of one week to one month and all sensitizing drugs require a few doses (more than one) to cause reactions. However, duration of the treatment is not a risk assessment parameter (Smith *et al.* 2009).

Reactive metabolites are almost always associated with IDRs (Smith & Obach 2005, 2006). The initial event is bioactivation of a drug to a reactive metabolite that forms a covalent bond (irreversible) nonselectively to proteins. In rare cases the reactive metabolite acts as a hapten, which can trigger through a covalent protein binding the immune system (the hapten hypothesis). In another hypothesis, the danger hypothesis, it is assumed that reactive metabolites covalently bond to cells causing cell stress or damage sometimes leading to a danger signal triggering the immune system to act (Park *et al.* 1998, Uetrecht 2003, Uetrecht 2007, Uetrecht 2008).

Reactive metabolites cannot be directly measured due to their reactivity, but their downstream products can be found from the excreta, usually as glutathione conjugates or their further metabolic products (e.g. mercapturic acids). These structures are indicative of the past reactivity. As all drugs forming reactive metabolites do not cause IDRs and high-dose drugs (>200 mg/day) seem to cause more reactions than low-dose drugs, it is assumed that the total body burden of reactive metabolites is the most important risk assessment parameter. The risks increase when the body reservoirs of detoxification substances (e.g. glutathione) are dangerously depleted because of the reactive metabolite production. For drugs dosed at less than 50 mg a day, excreted metabolites are generally considered of little or no concern unless the drugs contain very susceptible moieties (see 2.8.4, type C toxicity). With higher dosed drugs, the absolute mass of excreted reactive metabolite downstream products should be considered in risk assessment. General guidance encourages identifying all metabolites (any structure in urine and feces)

having at least 10 mg daily excretion in humans and determining whether reactive metabolites are involved in the reaction pathway. If there is a risk, one should ensure that the species used in safety assessments generate the reactive metabolite of concern at sufficient levels (Smith & Obach 2005, Smith & Obach 2006, Smith & Obach 2009, Smith *et al.* 2009).

### *2.8.4 Type C toxicity*

Type C (chemical) toxicity is quite similar to type B toxicity, but it arises more regularly with rapid onset, has a clear dose-response relationship, and can occur after a single high dose. Type C toxicity is normally caused by bioactivation of the drug to a reactive metabolite that directly, or after consuming detoxification reservoirs such as glutathione, reacts with macromolecules resulting in a rapid toxic response through direct damage to the cell or subcellular units. In the case of type C toxicity, compared to type B, the reactivity can be predicted (or rationalized) based on the chemical structure of the drug (Park *et al.* 1998, Smith *et al.* 2009, Smith & Obach 2009, Frederich & Obach 2010). Bioactivation can form metabolites undergoing redox cycling (e.g. quinones ↔ hydroquinones), deplete intracellular stores of reduction potential (e.g. reduced glutathione) and cause oxidative stress (after formation of reactive oxygen species), which can lead to cell death and tissue necrosis (Smith & Obach 2005, Testa 2009). The same risk assessment tool of 10 mg total daily body burden of metabolite is generally used as for type B toxicity (Smith & Obach 2005, 2009). The reactive metabolites seem to show selectivity to certain protein targets (Hanzlik *et al.* 2007, Hanzlik *et al.* 2009, Liebler 2008).

The toxic potential of many compounds is explained by their metabolic activation to electrophilic intermediates or free radicals. Certain structural alerts, potential toxicophores, are found frequently behind type C toxicity (Williams & Park 2003, Williams 2006, Guengerich & MacDonald 2007, Naito *et al.* 2007, Testa & Krämer 2009, Stepan *et al.* 2011). Potential toxicophoric groups and resulting toxicophores have been listed (Testa 2009, Testa & Krämer 2009):

- Aromatic systems oxidized to epoxides, quinones, quinone imines and quinone methides
- Aromatic ethynyl groups oxidized to ketenes
- Arylamines and arylamides oxidized to N-oxygenated species and nitrenium ions

- Nitroarenes reduced to N-oxygenated species and nitrenium ions
- Thiocarbonyl compounds oxidized to sulfenes
- Thiols oxidized to sulfenic acids leading to mixed disulfides with proteins
- Carboxylic acids forming acyl glucuronides
- (Hydroxymethyl)arenes forming sulfate esters
- Polyhalogenated alkyl groups reduced to radicals
- Polyphenols and quinones forming superoxide
- Carboxylic acids forming lipophilic residues via their coenzyme A conjugates
- Primary arylamines forming poorly soluble N-acetyl conjugates

One should remember that the most common chemical outcome of bioactivation is not a toxic reaction, but an inactivation reaction, usually via conjugation of a reactive metabolite with GSH or another detoxification system, e.g. hydrolysis of electrophilic epoxide with epoxide hydrolase (Testa & Krämer 2009, Stepan *et al.* 2011).

### *2.8.5 Type D toxicity*

Type D (delayed) toxicity reactions are long-term toxicities like carcinogenicity and teratogenicity. These adverse effects occur months, years or even decades after the treatment or exposure (Park *et al.* 1998, Park *et al.* 2000). Carcinogenesis can be caused by chemically reactive metabolites reacting with genetic material (mechanism similar to Type B and C), or through hormonal processes involved in cell growth and regulation (mechanism similar to Type A). Carcinogenicity has a dose-response relationship (Smith & Obach 2009, Frederick & Obach 2010). However, the duration of exposure is a 10 times more relevant risk factor than concentration or dose size. Another important factor is the age of the patient at the onset of long-term treatment with a potentially carcinogenic medication. It may take 30 years from exposure to the carcinogenic event, and thus, the younger the patient the greater the risk of actual carcinogenicity (Smith *et al.* 2009). There are over 50 known structural alerts (toxicophores) for mutagenicity and carcinogenicity (Enoch & Cronin 2010).

Teratogenicity also has similar mechanisms to Type A, B and C toxicity, including receptor mediation and oxidative stress. The mechanisms are mostly similar to Type A1 or A2 involving circulating metabolites. Exposure of the toxic agent has to occur during a critical developmental time period, such as gestation or childhood (Smith & Obach 2009, Frederich & Obach 2010).

## 2.9 Reactive metabolites

The formation of chemically reactive metabolites (metabolic activation, bioactivation) is one of the main reasons for attrition in drug discovery and early development due to their association with Type B, C and D toxicities.

### *2.9.1 Avoidance of reactive metabolite formation*

Since the early 2000s the industry has adopted an avoidance strategy to minimize the potential for reactive metabolite formation from their lead molecules and drug candidates. Toxicophoric functional groups may be excluded from drug design irrespective of their potential pharmacologic, pharmacokinetic or biopharmaceutical advantages (Baillie 2006, Shu *et al.* 2008, Kalgutkar & Dalvie 2015). In cases where toxicophores cannot be completely avoided because they are also pharmacophores and bioactivation suspected, medicinal chemists have a considerable toolbox to get rid of the potential liability (Stachulski *et al.* 2013):

- Introducing a minor substituent (e.g. fluorine atom) to block the metabolic activation pathway. This usually has minor effect on pharmacological properties, but efficiently redirects the metabolism.
- Introducing a steric hindrance to the vicinity of the bioactivation site (e.g. change N-atom to S-atom). This results in reorientation of the compound at the active site of metabolizing enzyme.
- Decreasing electron density in the nucleophilic center undergoing metabolic activation. Incorporation of e.g. a halogen atom or nitrile group close to the nucleophilic center withdraws electron density and lowers the probability of oxidation by CYP enzymes.
- Structural modification to create a metabolic soft spot and alternate metabolic pathway. Addition of a simple methyl group to or close to the bioactivation site may be sufficient to create an oxidation site away from the reactive metabolite formation.
- Replacing liable functionality by a similar, but more stable substituent, if the toxicophore is not the pharmacophore of the compound. For example, different aryl systems may need to be introduced to assure positive biopharmaceutical properties without bioactivation.

### 2.9.2 Experimental approaches to study bioactivation

Valid animal models would really be appreciated for bioactivation study, but such models are rare (Uetrecht 2006). A recent review summarizes only eight animal models for idiosyncratic drug reactions (Uetrecht & Naisbitt 2013). Most of the animal models presented have serious limitations and only one model shows the same IDR mechanism as humans. The facts that IDRs are rare in humans, adverse reactions are quite often minor (e.g. itching skin) and humans can develop an immune tolerance, make it very difficult to develop the same mechanistic animal models. Another important reason for the difficulty involved in animal model development is that animals may not produce at all (or not sufficiently) the reactive metabolite needed for the IDR to be triggered (Uetrecht & Naisbitt 2013). As noted in an *in vitro* comparison study of 55 compounds in humans and rat, the metabolism profiles can be very similar or very different (Pelkonen *et al.* 2009b).

Besides avoidance strategy, another default procedure is to use appropriate *in vitro* investigations at the discovery and lead optimization phases to identify potential metabolic liabilities. However, there are no straightforward correlations between reactive metabolite formation *in vitro* and idiosyncratic adverse drug effect. All drugs forming reactive metabolites *in vitro* do not cause adverse events *in vivo* (false positives) and vice versa (false negatives). Human *in vivo* metabolism may occur at a completely different site than *in vitro* (other than toxicophore) and lead to nonreactive metabolism. On the other hand, perfectly safe-looking molecules without known toxicophores may end up producing reactive metabolites through an unexpected metabolic route or catalyzing enzyme (Stachulski *et al.* 2013, Kalgutkar & Dalvie 2015). In some cases, metabolic activation might be only one of the possible mechanisms behind observed adverse events (Tang & Lu 2010).

The existence of false positives and multiple mechanisms raises a concern that too stringent *in vitro* cutoff limits for reactive metabolite formation lead to attrition of potentially great new medicines that would have been completely safe and would have had a significant impact on the treatment of serious human illnesses (Park *et al.* 2011a). The ever increasing sensitivity of analytical instruments is potentially leading to a situation where evidence of reactive metabolites can be detected for all investigated molecules in one or more *in vitro* test systems (Park *et al.* 2011b). This puts pressure on good scientific practices,

physiologically relevant testing systems and clinically relevant conditions used (Li 2009, Smith 2009, Park *et al.* 2011a).

Even though there are well-established toxicology practices for regulatory purposes (that have actually not changed in decades) the prediction of human drug toxicity is difficult. One of the main reasons is species difference as preclinical safety testing is based on laboratory animal experiments. Additionally, most animal models are designed for acute toxicity and do not represent mechanisms for rare adverse events (Uetrecht & Naisbitt 2013). Unique to human metabolites cannot be tested at this point and unique to human toxicity will not be detected (Li 2009). There is no solid experimental procedure for human idiosyncratic toxicity risk assessment beyond *in vitro* methods. The first true biological toxicity alert may be an adverse event in late development (clinical trial) or after approval (Park *et al.* 2011b).

The experimental approaches to find and identify reactive metabolites and to evaluate their significance can be divided into two categories: the ones that require synthesis of radioactive study compound and the ones that can be done with cold study compound. As synthesis of radioactively labeled compound is rather expensive, these studies are done in the later phases of drug development for advanced compounds. These studies are mostly inside the regulatory package of DMPK and ADME *in vivo* studies with radiolabeled compound. At the early discovery and lead selection phases only the cold compound is available and most of the studies are performed *in vitro*. Some of these studies include routine drug metabolism studies for each novel compound, some study samples are extracted from the routine study for additional examination, and some of the bioactivation investigations are triggered by the findings in the routine studies (Li 2009, Park *et al.* 2011a, Stachulski *et al.* 2013, Kalgutkar & Dalvie 2015).

*Studies with cold study compound:*

- Time-dependent CYP enzyme inhibition (*in vitro*)
- Smoking gun metabolite identification (*in vitro* and *in vivo*)
- Reactive metabolite trapping (*in vitro*)
- Quantitative reactive metabolite trapping (*in vitro*)
- Extrahepatic metabolism (*in vitro*)
- Acyl glucuronide kinetics (*in vitro*)

*Studies requiring radiolabeled study compound:*

- Covalent binding (*in vitro*)
- Smoking gun metabolite identification (*in vivo*)
- Plasma radioactivity recovery (*in vivo*)
- Mass Balance (*in vivo*)
- Tissue binding in QWBA (*in vivo*)

The most important *in vitro* approaches, covalent binding and reactive metabolite trapping of chemically reactive metabolites, are discussed separately after the following discussion of the other bullets.

The examination of potential effect on CYP activity is a typical and commonly automated procedure for new compounds in drug discovery. If reactive metabolites are produced in the test setup, covalent binding to the CYP enzyme may happen and lead to inhibition of the CYP enzyme by time-dependent inactivation. Time-dependent inhibition (TDI), also called metabolism-dependent inhibition (MDI), can be a starting point for further reactive metabolite investigations. Significant TDI together with high daily dose, covalent binding and GSH adduct formation may indicate potential risk for adverse events (Reese *et al.* 2011, Sakatis *et al.* 2012). However, TDI only is not a proof of reactive metabolite generation as there are other mechanisms of time-dependent inhibition as well (Li 2009, Park *et al.* 2011a, Stachulski *et al.* 2013, Kalgutkar & Dalvie 2015). There is considerable variation in the experimental design and data analysis of TDI studies which may bias the results and predictions from one laboratory to another (Stachulski *et al.* 2013).

Reactive metabolites are not usually detected as such; instead, their downstream products that are stable enough to survive until analysis are detected. These "smoking gun" metabolites include most importantly GSH adducts and mercapturic acids, but also dihydrodiols, acyl glucuronides, carboxylic acids, methyl catechols, and acetic acid. They can be found – and should be searched – with current analytical technologies from *in vivo* and *in vitro* samples (human and animal) routinely generated for the evaluation of properties of a new molecule (Bass *et al.* 2009) during the drug discovery phases. Some caution must be exercised if certain metabolites are only found from animal samples. The structures of these end-products should be elucidated to get indirect information concerning the identities of reactive metabolites and the site of bioactivation. As the isolation and concentration of these downstream products is difficult, artificial

methods to produce the reactive metabolites with *in situ* coulometric oxidation (Tong *et al.* 2010), electrochemical oxidation (Fashe *et al.* 2014) or biomimetically for large-scale production for standard use and NMR analysis (Lassila *et al.* 2015) might be useful. The quantitative content, plasma exposure and potential relevance of smoking gun metabolites can be measured from the samples of radiolabeled ADME studies (Obach *et al.* 2012, Stachulski *et al.* 2013, Kalgutkar & Dalvie 2015). Besides the liver, minor quantities of CYP enzymes as well as other drug metabolizing enzymes are also present in many organs. Sometimes it might be necessary to investigate tissue- or organ-specific bioactivation with appropriate extrahepatic preparations as organs have different proportions of reactive metabolite forming enzymes (Stachulski *et al.* 2013). A special case of reactive metabolites is acyl glucuronide, whose reactivity is based on the ability to direct transacylation and acyl migration to form reactive species towards amines (Bailey & Dickinson 2003). The measurement of kinetics of the acyl migration serves as an indication of acyl glucuronide reactivity (Walker *et al.* 2007). Analyses of half-lives of 21 acyl glucuronides in phosphate buffer, human serum albumin and fresh human plasma show that more stable acyl glucuronides are generally less toxic than rapidly migrating or transacylating ones (Sawamura *et al.* 2010).

Different LC/MS technologies are routinely used for the metabolite analysis and identification from a variety of *in vitro* and *in vivo* samples, including plasma samples form radiolabeled DMPK and ADME studies. Before analysis the plasma proteins need to be removed, usually by precipitating them with organic solvent. Measuring the total radioactivity of the plasma sample before precipitation and after complete washout of proteins gives plasma radioactivity recovery. Low recovery (e.g. below 85%) can be indication of covalent binding due to reactive metabolites. Further investigations may reveal covalent binding also in tissues, in addition to plasma (Stachulski *et al.* 2013). Quantitative whole body autoradiography (QWBA) study in rat can reveal potential tissue covalent binding, when radioactivity is seen in tissues for a prolonged time (Takakusa *et al.* 2008). However, the shortcoming is that the rodent data may not be relevant to humans due to species differences in reactive metabolite formation. Similarly to plasma recovery, low mass balance, i.e., recovery of radioactive excreta, provides indicative (but not unambiguous) evidence. However, there are no clear cutoffs for reactive metabolite alarms, as for example human mass balance recovery can be as low as 70% for various reasons (Roffey *et al.* 2007).

### *2.9.3 Reactive metabolite trapping*

Oxidations catalysed by CYP enzymes are the most common source of reactive metabolites, but also e.g. peroxidases, monoamine oxidases and flavin monooxygenases catalyze the formation of reactive metabolites. The reactivity is usually based on the electrophilic nature of these metabolites and they can react with numerous nucleophilic sites in the proteins, DNA and cell structures. The electrophiles are classified as soft and hard. They are reactive because their electrophilic center is electron-deficient. Hard electrophilic centers have high positive charge density while soft ones have lower positive charge density. Nucleophiles are electron rich at their nucleophilic center. Soft electrophiles like epoxides and quinones will react with soft nucleophiles, such as protein thiol and amino groups. Likewise, hard electrophiles, e.g. aldehydes and imines, react with hard nucleophiles, such as DNA bases or protein serine and threonine residues. Additionally to oxidation reactions, conjugation reactions may produce hard nucleophiles through spontaneous breakdown to carbonium or nitrenium ions.

**Table 4. Examples of hard and soft electrophiles and endogenous nucleophiles (from Stachulski *et al.* 2013), and nucleophilic trapping agents for both electrophile groups.**

| Type | Electrophiles | Endogenous nucleophiles | Trapping agents |
|---|---|---|---|
| Hard | Alkyl carbonium ions | Oxygen atoms of purine/pyrimidine | KCN |
| | Benzyl carbonium ions | Bases in DNA | Semicarbazide |
| | Iminium ions | Endocyclic nitrogens of purine bases in DNA | Methoxylamine |
| | Aldehydes | Oxygen atoms of protein serine and threonine residues | |
| Soft | Epoxides | Protein thiol groups | GSH and GSH |
| | Enones | Sulfhydryl groups of glutathione | derivatives |
| | Quinone imines and methides | Primary/secondary amino groups of protein | N-acetylcysteine |
| | Michael acceptors | lysine and histidine residues | Mercaptoethanol |

Table 4 summarizes examples of hard and soft nucleophiles, the respective electrophiles and chemical agents used to trap each class of electrophiles (Stachulski *et al.* 2013). Sometimes reactive metabolites are free radicals possessing an unpaired electron that can abstract a hydrogen atom from endogenous molecules, or they can add themselves to double bonds (Testa & Krämer 2009, Stachulski *et al.* 2013

Reactive metabolite trapping experiments are most frequently done with unlabeled drug incubated with human liver microsomes fortified with appropriate cofactors and reduced GSH. Glutathione is the natural reactive metabolite trapping agent having a concentration of approximately 10 mM in the human liver. GSH adduct formation can be used as a surrogate marker for reactive metabolites (Masubuchi *et al.* 2007). Different LC/MS methods are used for the detection of the glutathione adducts formed (Park *et al.* 2011a, Stachulski *et al.* 2013, Kalgutkar & Dalvie 2015). Distinguished exact mass and characteristic fragmentation pattern have been routinely used for years to aid the fast and automated detection of GSH adducts and identification of tentative structures (Samuel at al. 2003, Ma *et al.* 2008, Zhang & Yang 2008, Ma & Zhu 2009). As the instrumentation and detection algorithms continue to develop, more sophisticated and efficient methods are frequently being introduced (Rousu *et al.* 2009, Brink *et al.* 2014) to facilitate rapid identification of reactive metabolites and to help medicinal chemists to modify the lead molecule structures.

Glutathione is not able to catch all of the soft electrophiles, and additional trapping agents have been proposed (Baillie 2008). The most frequently used are N-acetyl cysteine (Xu *et al.* 2005) and mercaptoethanol (Baillie 2008). Also, different GSH derivatives have been introduced for qualitative, quantitative and semi-quantitative workout (Gan *et al.* 2005, Soglia *et al.* 2006, Ma & Chan 2010, Lentz *et al.* 2014). The most frequently used quantitative assessment of reactive metabolites utilizes $^{35}$S-labeled glutathione (Masubuchi *et al.* 2007, Takakusa *et al.* 2009). It should be kept in mind that true quantitation can only be reached with radioactivity detection. Radioisotopically labeled glutathione can, however, aid in the detection of minor adducts in LC/MS analysis (Yan & Caldwell 2004, Rousu *et al.* 2009). The typical experimental setups with liver microsomes for GSH adduct formation actually favor the generation of reactive metabolites and do not take into account alternative metabolism and detoxification pathways. Thus, one must be cautious when drawing conclusions from the experiments even if the experiments were quantitative in nature (Park *et al.* 2011a, Stachulski *et al.* 2013, Kalgutkar & Dalvie 2015).

Combination of *in vitro* and *in vivo* metabolism studies, extensive reactive metabolite trapping experiments with microsomal preparations, S9 fraction and especially hepatocytes are emphasized to get the full picture of bioactivation potential through reactive metabolism (Park *et al.* 2011a, Stachulski *et al.* 2013, Kalgutkar & Dalvie 2015). Quantitative [$^{35}$S]GSH trapping and TDI assay can be a useful combination to spot potentially highly reactive metabolites in the early

stages of drug discovery (Nakayama *et al.* 2011). Intended daily dose of the drug and total body burden of a potentially reactive metabolite should be considered in the quantitative risk evaluation. For example, there are no drugs currently marketed that cause idiosyncratic adverse events, if the dose is less than 20 mg/day (Kalgutkar & Didiuk 2009, Kalgutkar 2011). Additionally, less than 1 mg/day total body burden of a potentially reactive metabolite seems to be a safety limit in avoiding drug-induced toxicity (Gan *et al.* 2009, Humphreys 2011).

Hard nucleophiles can also be trapped with several agents. For qualitative LC/MS and quantitative LC/RAD work unlabeled and labeled potassium cyanide, respectively, have been successfully used (Argoti *et al.* 2005, Meneses-Lorente *et al.* 2006, Rousu & Tolonen 2010, Barbara *et al.* 2012). Other frequently used trapping agents for quantitative and qualitative work are semicarbazide (Rousu *et al.* 2009, Miura *et al.* 2010) and methoxylamine (Baillie 2008). Additionally, there have been proposals of peptide trapping agents capable of simultaneously trapping both hard and soft electrophiles (Yan *et al.* 2007, Mitchell *et al.* 2008).

Hard electrophiles reacting with nucleic acids may lead to genotoxic and carcinogenic outcomes. Dobo *et al.* (2009) have generalized a risk assessment model for potentially genotoxic metabolites based on total body burden of suspected risky metabolites. The risk of genotoxicity for any metabolite is low,

- if the total body burden of a metabolite in rodents is over 10 mg/kg/day and no tumorigenic response is detected in the carcinogenicity study, or
- if the total body burden of a metabolite in humans is less than 1.5 μg/day, or
- if the rodent total body burden of a metabolite exceeds that of human body burden by over 1000x.

### *2.9.4 Covalent binding*

In a typical covalent binding study, a radioisotopically labeled test molecule is incubated with human liver microsomes and appropriate cofactors to allow metabolism. If reactive metabolism occurs, some of the reactive intermediates may covalently bind to the microsomal proteins, which is measured from the precipitated and washed protein pellet using a radioactivity detector. The procedure was semi-automated already 10 years ago (Day *et al.* 2005). If careful tritium labeling to a metabolically stable part of the molecule is used, it can be employed for relatively many lead candidates at the later phases of drug discovery

(Samuel *et al.* 2003). However, the covalent binding study is typically performed for a few drug candidates at the early development phase (Stachulski *et al.* 2013). It has actually been shown that there is a good correlation between *in vitro* covalent binding and maximum *in vivo* covalent binding, if the model is based on the free fraction of plasma AUC (Masubuchi *et al.* 2007).

After the seminal perspective article of Evans *et al.* (2004) from Merck, the majority of pharmaceutical industry adopted a threshold value for microsomal covalent binding of 50 pmol drug-equivalent/mg protein. The 50 pmol/mg is not a definitive upper limit for covalent binding, but a drug developer should be aware of and consider the potential for reactive metabolites, bioactivation and associated adverse events for molecules with higher covalent binding value. Also, alternative metabolic detoxification and clearance routes as well as projected therapeutic dose should be considered before making medicinal chemistry decisions (Evans *et al.* 2004). Recently, it was determined that 65% of compounds having greater than 200 pmol/mg covalent binding are likely to be hepatotoxic *in vivo* (Sakatis *et al.* 2012). For medicinal chemistry purposes a good approach would be a combination of covalent binding and reactive metabolite trapping of lead series compounds to find the most potent and safe drug candidate (Hagmann 2008, Stachulski *et al.* 2013).

### *2.9.5 Daily dose considerations*

Idiosyncratic drug reactions are not thought to be linearly dose-dependent. Careful examination of 230 oral drugs from pharmaceutical databases and adverse event registers has shown that risk for e.g. idiosyncratic drug-induced liver injury (DILI) increases when daily dose exceeds 50 mg/day (Lammert *et al.* 2008). When the microsomal covalent binding data is put together with clinically used or projected daily dose, the prediction of which compounds are likely to cause idiosyncratic toxicity is improved (Obach *et al.* 2008b). Several industrial research groups changed microsomes to hepatocytes to add additional metabolism and detoxification routes to the bioactivity study setup and achieved good discrimination between the two groups (tox/non-tox), but some overlap still remained, predicting false positives and negatives (Nakayama *et al.* 2009, Usui *et al.* 2009, Baumann *et al.* 2009). Even if the results and models presented were useful for the industry, clear threshold values for dose/covalent binding relationship could not be announced for guidance. Each study also had a common limitation in that the study set was quite low: 20–40 compounds.

The most comprehensive study so far to discriminate between toxic and non-toxic compounds included 223 marketed drugs, 51% associated with clinical hepatotoxicity and 49% non-hepatotoxic ones. The study investigated daily clinical dose, time-dependent inhibition of CYP enzymes, GSH trapping (microsomes) and covalent binding (microsomes), and came out with rationally justified decision-making parameters (Reese *et al.* 2011, Sakatis *et al.* 2012):

- If the clinical daily dose is less than 100 mg, proceed with development as 76% of compounds are non-hepatotoxic ($p < 0.0001$)
- If the clinical daily dose is at least 100 mg and cofactor-dependent TDI of any CYP enzyme decreases over 5-fold in IC50, terminate development as 94% of the compounds are hepatotoxic ($p < 0.0001$)
- If the clinical daily dose is at least 100 mg and any intensity of cofactor-dependent or independent GSH adduct is observed, terminate development, as 80% of the compounds are hepatotoxic ($p < 0.0001$)
- If the clinical daily dose is at least 100 mg and covalent binding is over 200 pmol eq/mg protein, terminate development, as 100% of the compounds are hepatotoxic ($p < 0.0001$).

TDI and GSH trapping are first line studies conducted in the early discovery, and covalent binding a second line study for advanced compounds. The intrinsic limitations include lack of hard electrophiles and acyl glucuronides in the GSH trapping and potentially investing company resources in a false negative compound to be terminated later and early termination of 10% of the non-hepatotoxic compounds (false positives) due to strict cut-off. This early termination strategy formulated as a comprehensive decision tree is routinely applied at GlaxoSmithKline to provide guidance to drug discovery project teams aimed to avoid potentially hepatotoxic compound (Sakatis *et al.* 2012).

The AstraZeneca approach combines covalent binding and daily dose as covalent binging daily burden (CVB burden) to a panel of five *in vitro* toxicity assays (*In vitro* Panel) to get their Integrated *In vitro* Hazard Matrix to predict idiosyncratic drug reactions (Thompson *et al.* 2011, Thompson *et al.* 2012). In the study set of 36 marketed or withdrawn oral drugs the Hazard Matrix correctly predicts 93% of IDR positive compounds and 100% of IDR negative ones. The *In vitro* Panel contains inhibition of BSEP and Mrp2 transport activity, cytotoxicity in HepG2 cells and cytotoxicity in THLE-Null and THLE-3A4 cells. The use of THLE-3A4 cells expressing CYP 3A4 and THLE-Null cells not expressing CYPs

for DILI was further validated in a study with 103 compounds (Gustafsson *et al.* 2013). The usefulness of cytotoxicity assays for the identification of bioactivation liabilities have also been highlighted in a large database study of Pfizer compounds (Shah & Greene 2014) and for THLE (Greene *et al.* 2010) and HepG2 (Choi *et al.* 2015) in particular. The CVB Burden is calculated by determining the fraction of metabolism leading to covalent binding in human hepatocytes multiplied by maximum prescribed daily dose. Intestinal absorption and fraction metabolized of the dose were set at unity to reflect worst-case scenario. It was concluded that compound failing in two or more *in vitro* toxicity assays and compounds having a covalent binding daily burden of more than 0.9 mg/day possessed hazard for IDRs (Thompson *et al.* 2012). This approach brings cellular activities mechanistically linked to risk of IDRs into the bioactivation experiments. It potentially fixes the limitation of previous approaches that some drugs cause IRDs through mechanisms not involving metabolic activation. The Integrated *In vitro* Hazard Matrix is currently an integrated part of AstraZeneca's drug discovery and development process (Thompson *et al.* 2012).

Pfizer has also recognized that complete avoiding of reactive metabolites may be a useful and pragmatic approach to migitatethe risk of idiosyncratic toxicity, but only as a starting point. In addition to reactive metabolite screening, more integrated paradigm for the identification of chemical hazards is needed. The detailed assessment of reactive metabolite formation should be combined with information of cellular death (e.g. cytotoxicity), BSEP inhibition and mitochondrial toxicity according to Pfizer (Stepan *et al.* 2011).

Reactive metabolite formation should be regarded as marker of bioactivation and not as marker of definitive toxicity. The above-described *in vitro* studies can only identify potential hazards even if intended daily dose is taken into consideration (Park *et al.* 2011a). All the compiled bioactivation data should be placed in a broader risk-benefit assessment context (patient population, indication area, duration of treatment, mechanism of action, route of administration etc.) before actual decision-making concerning the continuation or termination of a certain molecule (Kalgutkar & Didiuk 2009, Sakatis *et al.* 2012). Until there is a better understanding of mechanisms behind idiosyncratic toxicities, decreasing the dose size and associated reactive metabolite body burden by improving potency and pharmacokinetics of the parent drug will remain as important goals in drug discovery and development (Stepan *et al.* 2011).

# 3 Aims of the present study

Ospemifene is a novel nonsteroidal selective estrogen receptor modulator (SERM) having both agonist and antagonist activity at estrogen receptors (Wurz *et al.* 2013, Pinkerton & Thomas 2014). It was recently approved by the US Food and Drug Administration (Feb 2013, trade name Osphena) and the European Medicines Agency (Nov 2014, trade name Senshio) for the treatment of vulvar and vaginal atrophy in postmenopausal women with moderate to severe dyspareunia. The approved dose in both authorizations was once daily oral tablet with 60 mg ospemifene of which efficacy and safety have been thoroughly investigated (most recent articles: Simon *et al.* 2014, Portman *et al.* 2014, Wurz *et al.* 2014, Nappi *et al.* 2015). A summary of the development milestones of ospemifene (Elkinson & Yang 2013) and a review and meta-analysis of the published clinical trials (Cui *et al.* 2014) have also been reported.

The objectives of the study were to characterize the metabolic properties and drug interaction potential of ospemifene. Additionally, the aim was to provide metabolism information for the drug development of ospemifene, including assessment of metabolism-related clinical safety. The specific aims were:

- To identify the human *in vitro* and *in vivo* metabolites of ospemifene for biotransformation pathways.
- To determine the main human metabolites of ospemifene *in vitro* and *in vivo*.
- To explore the metabolism of ospemifene in different laboratory animals *in vitro* and *in vivo*.
- To find out if the nonclinical animal exposure of main ospemifene metabolites has been enough to cover the human dose.
- To find out the most important metabolic enzymes catalyzing ospemifene metabolism.
- To investigate the effects of ospemifene and its metabolites as perpetrator drugs on drug metabolism mediated by CYP enzymes in human.
- To investigate the effects of CYP enzyme inhibitors and inducers on the metabolism of ospemifene and its metabolites as victim drugs.
- To find out the *in vivo* drug interaction potential of ospemifene.

# 4 Materials and methods

Detailed descriptions of the materials and methods used in this study are found in the original articles I, II, III, IV and V as referenced in Table 5.

**Table 5. The original sources of the materials and methods used in this study.**

| Materials and methods | | Original article |
|---|---|---|
| Materials | Chemicals, MS-TOF and MS/MS experiments | I, II |
| | Chemicals, *in vitro* incubations | II, III, IV, V |
| | Chemicals, LC/MS measurements | II, V |
| | Human liver samples | II, III, IV, V |
| | Animal (rat, dog, monkey) liver samples | V |
| | cDNA expressed human CYPs | II |
| | Human plasma samples | II |
| | Animal (mouse, rat, dog, monkey) plasma samples | V |
| | Human hepatocytes | III |
| Methods | MS-TOF and MS/MS experiments | I, II |
| | Human liver homogenate preparations and incubations | II, V |
| | Human liver microsome preparations and incubations | II, III, IV |
| | Animal liver homogenate preparations and incubations | V |
| | Incubations with cDNA expressed human CYPs | II |
| | N-in-one CYP inhibition assay | III, IV |
| | Kinetic CYP inhibition assays | III, IV |
| | CYP induction study with isolated human hepatocytes | II |
| | Sample preparation for *in vitro* and *in vivo* metabolite analysis | II, V |
| | LC/MS methods for ospemifene and metabolites | II, IV, V |
| | LC/RAD analysis of ospemifene and metabolites | II |
| | *In vivo* drug interaction studies | III, IV |
| | Pharmacokinetic parameter calculations | III, IV |

## 4.1 Chemicals (I–V)

Ospemifene and synthetic metabolites of ospemifene (3-, 4, and 4'-hydroxyospemifene and ospemifene carboxylic acid, M4, M1, M2 and M3, respectively) were provided by Hormos Medical Corp. The analytical solvents and mobile phase additives used for the metabolism and drug interaction analyses were HPLC or LC/MS grade and were obtained from commercial suppliers. Ultra-pure grade laboratory water was purified with commercial purification systems. All other reagents used in the drug interaction studies were obtained from commercial providers at the highest purity reasonably available.

## 4.2 Human and animal liver preparations (II–V)

Human liver preparations were pooled from 7–10 human livers (HL20–24, HL28–32), depending on original article II–V, obtained from Caucasian kidney transplantation donors (age 21–62 years, four females and six males). The Ethics Committee of the Medical Faculty, University of Oulu, has approved the collection of surplus human tissues for investigational purposes. Detailed donor information and preparation of homogenates and microsomes were published in Turpeinen *et al.* (2007). Briefly, normal-looking liver tissue was homogenized in phosphate buffer (homogenate used as such), microsomes were separated by differential centrifugation (Pelkonen *et al.* 1974) and the final microsomal pellet was suspended in phosphate buffer. The accurate protein content was measured by the method of Bradford (1976).

Animal liver homogenates (V) were prepared similarly to human liver homogenates and the liver samples were left-over tissue from untreated animals used as controls. The respective homogenates were pooled from livers of two beagle dogs, four Sprague-Dawley rats and four Cynomolgus monkeys. The monkey homogenate was actually reconstructed from 10,000 g supernatant and microsomal suspension.

### *4.2.1 Metabolism in liver homogenates or microsomes (II, V)*

Ospemifene, 4- or 4'-hydroxyospemifene was incubated with human or animal liver homogenates or microsomes in the presence of appropriate cofactors: NADPH and UDPGA were used for both microsomes and homogenates (II, V), and additionally, PAPS and GSH were used for homogenates in II. Typical incubation time was 60 min and the nonmetabolized 0 min samples were stopped before the metabolism reactions were started by adding the cofactors. A parallel incubation without cofactors was used to investigate spontaneous metabolism. Variable concentrations of the study compound were employed depending on the specific purpose of the incubation.

### *4.2.2 CYPs involved in ospemifene metabolism (IV)*

Human liver microsome incubations with one concentration of ospemifene as substrate were conducted using 12 different CYP selective inhibitors (Pelkonen *et al.* 2008a) at five logarithmic concentrations and compared to control reactions

(Turpeinen *et al.* 2005). The formations of 4- and 4'-hydroxyospemifene were measured as activities. $IC_{50}$ values were determined for each inhibitor and both activities graphically from concentration-activity curves. The selection of ospemifene concentration was based on the results with ospemifene and recombinant CYP enzymes (II). CYP enzyme contributions for 4- and 4'-hydroxyospemifene (as substrates) were obtained similarly.

Based on the results with 12 CYP selective inhibitors additional incubations were designed with selected CYP-specific inhibitors and ospemifene in human liver microsomes. Three low concentrations of ospemifene were used together with two different concentrations of inhibitors thioTEPA (CYP2B6), sulfaphenazole (CYP2C9), fluconazole (CYP2C19) and itraconazole (CYP3A4).

### *4.2.3 CYP inhibition assays (III)*

The *in vitro* inhibition potential of ospemifene and its main metabolites 4- and 4'-hydroxyospemifene towards CYP enzymes was assessed using an N-in-one (cocktail) approach described and validated elsewhere (Turpeinen *et al.* 2005, Turpeinen *et al.* 2006, Tolonen *et al.* 2007). Five logarithmic concentrations of ospemifene substrates were used to obtain approximate $IC_{50}$ values. The concentrations of CYP-selective substrates were at or under the respective Michaelis-Menten coefficients. The enzyme activities of cocktail incubations were compared to control activities.

Based on the cocktail inhibition results, additional kinetic inhibition studies of ospemifene (four concentrations) toward CYPs 2B6, 2C8, 2C9, 2C19 and 2D6 were conducted using appropriate substrates separately in three concentrations ($K_m/2$, $K_m$ and $4*K_m$). Dixon, Lineweaver-Burke, Hanes and Hofstee plots were used to determine the type of inhibition for each CYP enzyme.

## 4.3 Human hepatocytes and recombinant CYP enzymes (II, III)

Supersomes (cDNA expressed human CYP enzymes) were obtained from BD Biosciences (Bedford, MA, USA). The metabolism incubations of ospemifene with Supersomes for CYPs 1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A5 were conducted under linear velocity conditions according to the manufacturer's instructions (II).

Freshly isolated human hepatocytes were obtained from UK Human Tissue Bank (Leicester, UK). The detailed CYP induction assay for the potential of

ospemifene to act as CYP inducer is described in III. Briefly, hepatocytes were preincubated and then applied with three separate concentrations of ospemifene for 48 h in cell culture before adding separately CYP-specific model substrates for inducible CYPs: 7-ethoxyresorufin for 1A2, bupropion for 2B6, diclofenac for 2C9, S-mephenytoin for 2C19 and testosterone for 3A4. All model activities were measured after 1 h incubation using standard operating procedures established earlier. No RNA measurements were conducted. Parallel cell cultures with model inducers for CYPs 1A2 (omeprazole), 2B6 (phenobarbital), and 2C9, 2C19 and 3A4 (rifampicin) served as references.

## 4.4 Plasma and serum samples for metabolite analysis (II, V)

The human plasma samples analyzed (II) were from an open-label, single dose human ADME study with 60 mg ospemifene with and without 20.2 MBq tritium label on six postmenopausal volunteers (Bryson *et al.* 2005). The analyses of human and animal samples after a dose of tritium-labeled ospemifene were performed by commercial laboratories with liquid scintillator counting (LSC) and their metabolite identification was based on retention time comparison to co-injected metabolite standards.

Human steady state serum and the mouse, rat, dog and cynomolgus monkey steady state serum and plasma samples analyzed (V) were taken from ospemifene preclinical and clinical studies conducted during the development in different commercial laboratories. The initial sample analysis was also performed by different commercial parties with varying analytical methodologies and the pharmacokinetic parameters used are taken from their reports.

## 4.5 Analytical conditions

### *4.5.1 Sample preparation (II–V)*

The *in vitro* samples (II–V) were generally treated the same way regardless of the incubation type. The *in vitro* incubation samples (stopped by precipitation before freezing) were thawed, shaken and centrifuged before collecting the supernatant for analysis. Human (II) and animal (V) plasma samples were thawed and precipitated, and the supernatants were evaporated before reconstitution for

analysis. Human and animal serum samples (V) were thawed and solid phase extracted, and the supernatants evaporated before reconstitution for analysis.

### *4.5.2 MS and LC/MS methods (I–V)*

The high-resolution MS fragmentation experiments (I, II) and LC/MS analyses (II, III, V) were conducted with two separate Micromass LCT time-of-flight (TOF) mass spectrometers and a Micromass LCT Premier XE TOF mass spectrometer. The unit resolution MS/MS experiments (I, II) and LC/MS/MS analyses (II–V) were conducted with Micromass Quattro II triple quadrupole mass spectrometer, Waters Quattro Premier triple quadrupole mass spectrometer and Thermo Finnigan LCQ Deca ion trap mass spectrometer. All experiments were performed in positive ion electrospray ionization mode. The MS and MS/MS experiments were performed with diluted reference compounds by direct infusion to the mass spectrometer.

The chromatography in LC/MS and LC/MS/MS analyses (II–V) were performed with Waters Acquity UPLC, Waters Alliance 2690 or Waters Alliance 2694 liquid chromatography systems equipped with autosampler, vacuum degasser and column oven, and Waters 996 PDA or Waters Acquity PDA detector. The analytical columns varied, but mainly 2.1*50 mm small particle size Waters SymmetryShield RP 18, Waters XBridge Shield RP18 and Waters BEH C18 were used for final analysis, with acidified water and methanol as eluents.

The analytical equipment (MS and HPLC instruments and HPLC columns) evolved tremendously during the long time period over which ospemifene analyses were performed. Thus, the instrumentation setup changed from analysis to analysis, but simultaneously, the mass spectrometric and chromatographic performance was better and better. When developing new methods for ospemifene analysis, old samples and reference standards were used to assure similar chromatography (order of eluted peaks) and the identification of metabolites not having reference standards.

## 4.6 Clinical interaction studies

### *4.6.1 Ospemifene as perpetrator drug (III)*

Three clinical interaction studies were conducted to investigate the potential *in vivo* effect of ospemifene to CYP enzyme mediated drug metabolism of concomitantly administered drugs. All studies were open-label, two-period, single center, balanced, crossover, Phase I pharmacokinetic studies in healthy white Finnish postmenopausal women. N was 12–16 depending on the study and the age range of the study volunteers was 50–71 years. In each study, one of the study periods was the pretreatment of subjects with once-daily oral 60 mg ospemifene for 7–12 days depending on the study before administering the single dose of concomitant medication. Equally in each study, the second study period consisted of a single dose of the concomitant medication without ospemifene pretreatment.

In study 1, the concomitant medication was 10 mg rasemic warfarin. Blood samples were collected in both study periods 120 h after warfarin administration and plasma S- and R-warfarin (S-warfarin metabolized by CYP2C9) concentrations were determined. In study 2, the concomitant medication was 150 mg bupropion. Blood samples were collected in both study periods 96 h after bupropion administration and plasma bupropion and hydroxybupropion (metabolism mediated by CYP2B6) concentrations were determined. In study 3, the concomitant medication was 20 mg omeprazole. Blood samples were collected in both study periods 8 h after omeprazole administration and plasma omeprazole, 5-hydroxy-omeprazole (metabolism mediated by CYP2C19) and omeprazole sulfone (metabolism mediated by CYP3A4) concentrations were determined. All analyses were performed using validated LC/MS/MS methods.

### *4.6.2 Ospemifene as victim drug (IV)*

Two clinical studies were conducted to investigate the potential *in vivo* effect of concomitantly administered drugs on the CYP enzyme mediated drug metabolism of ospemifene. Both studies were open-label, randomized, three-period, crossover, pharmacokinetic studies in healthy white Finnish postmenopausal women. N was 12 or 19, depending on the study, and the age range of the study volunteers was 48–80 years. In both studies, two study periods were the pretreatment of subjects with once-daily oral concomitant medication for 5–6 days depending on the concomitant medication before administering the single

oral dose of 60 mg ospemifene. The concomitant medication continued for a total of 8 days. The third study period in both studies consisted of a single 60 mg dose of ospemifene without pretreatment.

In the first study, the concomitant medication was either 600 mg rifampicin (strong CYP3A and CYP2C9 inducer) or 200 mg ketoconazole (CYP3A4 inducer), and in the second study, 200 mg fluconazole (1st day 400 mg, CYP3A, CYP 2C9 and CYP 2C19 inhibitor) or 40 mg omeprazole (CYP2C19 inhibitor). In the second study, the study subjects were genotyped as extensive CYP2C9 and CYP2C19 metabolizers. In all study periods, the blood samples were collected 96 h after ospemifene administration. Plasma concentrations of ospemifene, 4-hydroxyospemifene and 4'-hydroxyospemifene were determined by Hormos Medical Ltd (Turku, Finland) with a validated LC/MS/MS method using AB Sciex API4000 Q-trap instrument.

# 5 Results and discussion

Detailed descriptions of the results achieved in this study are found in the original articles I, II, III, IV and V. Major results are presented in Table 6.

**Table 6. The summary of major results of the study and their respective original articles.**

| Results | | Original article |
|---|---|---|
| Metabolism | | |
| | MS/MS fragmentation of ospemifene and its main metabolites | I, II |
| | Identification of human *in vitro* metabolites of ospemifene | II |
| | Determination of major human ospemifene metabolites as 4- and 4'-hydroxyospemifene | II, V |
| | Identification of further metabolites of 4- and 4'-hydroxyospemifene | II |
| | Identification of human *in vivo* metabolites of ospemifene | II, V |
| | Determination of 4- and 4'-hydroxyospemifene as major human *in vivo* ospemifene metabolites, 4-hydroxyospemifene being clearly the most important one | II, V |
| | Identification of animal metabolites of ospemifene *in vivo* and *in vitro* | V |
| | Determination of *in vivo* animal exposure of 4- and 4'-hydroxy-ospemifene as adequate | V |
| Interactions | | |
| | Production of 4- and 4'-hydroxyospemifene by cDNA expressed human CYP enzymes: major isoforms 2B6, 2C9, 2C19 and 3A4 | II |
| | Determination of ospemifene and its major metabolites as weak *in vitro* inhibitors of CYP isoforms 2B6, 2C8, 2C9 and 2C19 at clinically relevant concentrations | III |
| | Determination of *in vitro* CYP enzyme induction potential of ospemifene as negligible | III |
| | Clinical study to find the insignificant effect of ospemifene to pharmacokinetics of selected CYP enzyme substrates | III |
| | Determination of CYP isoforms 2C9, 2C19, 2B6 and 3A4 as the main *in vitro* catalysts for the production of 4-hydroxyospemifene and 3A4 for 4'-hydroxyospemifene by using specific CYP enzyme inhibitors | IV |
| | Determination of CYP3A4 enzyme responsible for the further metabolism of 4- and 4'-hydroxyospemifene *in vitro* | IV |
| | Clinical study on the effect of selected CYP enzyme inhibitors and inducers on pharmacokinetics of ospemifene and 4-hydroxyospemifene confirmed the importance of CYP3A4 to ospemifene metabolism | IV |

## 5.1 Metabolism of ospemifene

Ospemifene (Figure 7) was initially discovered as one of the main metabolites of the antiestrogen drug toremifene (Sipilä *et al.* 1990, Berthou & Dreano 1993). As toremifene and its paragons clomiphene and tamoxifen have extensive oxidative metabolism to O-linked side chain and 3-, 4-and 4'-positions of the triphenylethylene system (Mazzarino *et al.* 2013), similar metabolites for ospemifene were presumed as well. In fact, ospemifene carboxylic acid (M3) was already found as a toremifene metabolite (Sipilä *et al.* 1990). When development of ospemifene was initially started for the treatment and prevention of osteoporosis (DeGregorio *et al.* 2000, Qu *et al.* 2000), several presumed metabolites were already available as synthetic standard, including the actual main metabolite. However, the reported Phase I studies of ospemifene in male volunteers (single high dose up to 800 mg) and postmenopausal women (repeated daily dose up to 200 mg) only contain pharmacokinetics for ospemifene and no information about the metabolites (DeGregorio *et al.* 2000).

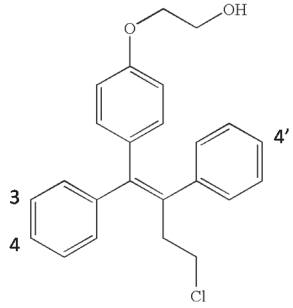


**Fig. 7. The structure of ospemifene.**

The *in vitro* and *in vivo* metabolism investigations reported in this study have been conducted over a period of about 10 years since 2000. As described in Materials and methods (4.5 Analytical conditions), the methodology has evolved over time, and additionally, some of the substudies have been analyzed by commercial third parties. Taking all the samples generated and analyzed for regeneration and reanalysis in a single batch would not change the main messages. Nevertheless, we would certainly gain new information and insight about the minor metabolites of ospemifene, but the clinical relevance of such information would remain scarce.

The study of ospemifene metabolism concentrated on human metabolism. First, human *in vitro* and *in vivo* metabolites were identified and

biotransformation pathways proposed based on the identifications. Secondly, circulating metabolites were quantitatively determined, main metabolites were confirmed and their relative abundances in comparison to ospemifene and all ospemifene-related material were solved. Thirdly, the production of main metabolites in different animals species was studied *in vitro* and *in vivo*, and finally, the animal exposures of active metabolites were determined.

### *5.1.1 Identification of ospemifene metabolites in human (I, II)*

Altogether nine significant metabolites listed in II, Table 1, were identified from human liver homogenate and microsome incubations and from plasma samples after 60 mg oral dose. The identifications were based on accurate mass data obtained with TOF-MS instruments and fragmentation with MS/MS instruments. The authentic standards were available for 4- and 4'-hydroxy-ospemifene (M1 and M2, respectively) and for ospemifene carboxylic acid (M3). The MS/MS fragmentation pathways are presented for ospemifene and metabolites M1 and M3 in I, Figures 3 and 6, and II, Figure 1. Most of the metabolites formed both sodium adducts and protonated molecules in the electrospray ionization source decreasing ionization efficiency, and thus sensitivity of the analysis when single ion or single reaction was monitored. Additionally, the ionization as sodium adduct might be non-reproducible based on the varying sodium content of the laboratory conditions. The sodium adduct of ospemifene carboxylic acid (M3) showed remarkable stability in MS/MS experiments compared to sodium adduct of ospemifene (I, Figures 1 and 5), probably due to side chain modification in M3. A side chain hydroxylated metabolite, M11, was discovered at high abundances in certain incubations, but the results were very difficult to reproduce. It might be that the M11 metabolite was unusually sensitive to the sodium content of the experimental setup and once the conditions were favorable the MS response was abnormally high. As the M11 was not found from *in vivo* samples, it was not relevant to pursue the unequivocal explanation.

In addition to parahydroxylated metabolites M1 and M2 (4- and 4'-hydroxylation, respectively), and side chain oxidated metabolites M3 and M11 (carboxylic acid and side chain dihydroxyl), three other significant oxidative metabolites and two glucuronidations were discovered. The oxidations were 3,4-dihydroxyospemifene (M6), a 4-hydroxyl-hydro-ospemifene (M9) and 4-hydroxyospemifene carboxylic acid (M10), and the glucuronidations were direct ospemifene O-glucuronide and a glucuronide of 4-hydroxyospemifene (site of

glucuronidation not known). Additionally, four metabolites were found from human and animal plasma samples after radioactive dose, namely M4 (3-hydroxyospemifene), M5 (3-hydroxyospemifene carboxylic acid, animal only), M7 (O-desalkylospemifene, human urine only) and M8 (4-hydroxy-O-desalkylospemifene). These metabolites were identified only based on retention time comparison to synthetic compounds (i.e., no TOF-MS or MS/MS data available). The hydro metabolite (M9) is interesting, as it likely originates through hydration of the central double bond. Such metabolites are actually known for closely related structures of clomiphene and toremifene (Mazzarino *et al.* 2013). M9 is also the only metabolite fragmenting a phenyl ring without oxygen (m/z 297), which supports the theory of modification in the central double bond.

The most abundant human metabolites in the *in vitro* incubations were 4- and 4'-hydroxyospemifene (M1 and M2), representing up to 75% and 16% of all metabolite peak areas, respectively. The ratio of M1:M2 was about 5:1 in all incubations. The same ratio was detected in human *in vivo* samples after quantification as well. All other discovered metabolites including the glucuronides were minor ones, representing up to a few percentages of all metabolites, except for the 4-hydroxy-hydro metabolite (M9) having up to 10% abundance at the highest incubation concentrations. The human *in vitro* main metabolite abundances are presented in II, Figure 3. The metabolism of the hydroxylated main metabolites was further studied by incubating those (synthetic metabolites) with human liver homogenates. M1 (4-hydroxyl) was found to metabolize to a glucuronide conjugate and to 3,4-dihydroxyospemifene (M6), and M2 (4'-hydroxyl) to a hydroxylated metabolite, but the abundance was too low to enable further structural elucidation.

### *5.1.2 Biotransformation pathways of ospemifene (II)*

The principal human circulating metabolites of ospemifene were proposed based on these results and presented in II, Figure 2. The primary pathway is 4-hydroxylation of ospemifene (M1), which can undergo further oxidative biotransformations at 3-position (dihydroxyl M6), side chain (carboxylic acid M10) and presumably to central double bond (hydration M9), and glucuronidation to one of the hydroxyl groups. The secondary metabolic pathway is 4'-hydroxylation (M2), which is unlikely to undergo extensive further metabolism even if a few further metabolites were sporadically found. Additional

minor biotransformation pathways include 3-hydroxylation (M4), side chain carboxylation (M3) and glucuronidation of ospemifene.

It is noteworthy that glutathione metabolites or significant levels of other worrying metabolites were not detected in human *in vitro* or in human plasma and urine. Minute amounts of reduced hydroxylated metabolites were, however, observed in some experiments, but the levels were too low to ascertain they were quinone methides. On the other hand, especially rat and mouse produced high amounts of hydroxyquinone metabolites (V, Table 1). However, the doses were extremely high compared to humans (300–2000 mg/kg in animals vs. $\leqslant$1 mg/kg in human). It is likely that intrinsic glutathione would easily detoxify quinones in humans, if they emerged. Moreover, it has been shown that closely related tamoxifen and toremifene both form unusually stable (non-reactive) quinone methides after 4-hydroxylation (Fan *et al.* 2000), which might be the case with possible ospemifene quinone metabolites as well. The formation of toremifene and tamoxifen quinone methides are considered not to have significant contribution to their cytotoxic or genotoxic effects (Fan *et al.* 2000), and the same is likely to hold true with ospemifene, if such metabolites were generated and GSH was depleted from the liver for some reason.

### *5.1.3 Circulating human metabolites (II)*

The quantitative plasma AUC values for ospemifene and eight most abundant metabolites measured after a single oral dose of 60 mg tritium-labeled ospemifene are presented in II, Table 2. The liquid scintillator counted AUC values for the metabolites were calculated as ospemifene equivalents by Covance Clinical Research Unit (Leeds, UK). Altogether ten circulating metabolites were found from human plasma. The main ospemifene metabolite was found to be 4-hydroxyospemifene (M1) representing about 15% of circulating ospemifene-related material (parent was 65%) and over 40% of all circulating metabolites. The AUC ratio of M1 and parent was about 22%. For all other metabolites, the metabolite-parent ratio remained below 8% and the share of all ospemifene-related material below 5%. The other identified metabolites included M2 (4'-hydroxyospemifene), M3 (ospemifene carboxylic acid), M4 (3-hydroxy-ospemifene), M8 (4-hydroxy-O-desalkylospemifene) and M10 (4-hydroxyospemifene carboxylic acid). M4 and M8 were so minor that they were not listed in II, Table 2.

In addition to identified metabolites there were four relatively significant metabolites detected in the radiochromatogram, but they were not identified due to lack of coeluting reference standards. Their AUC was at the same level or higher than 4'-hydroxyospemifene (M2) and they were quite polar metabolites, as they eluted before all the available reference metabolites. As no MS data was available for these metabolites and the chromatographic data is noncomparable, their identity remains speculative. Nonetheless, six previously detected human metabolites not having synthetic standards, namely ospemifene glucuronide and glucuronides of M1 and M2 as well as oxidative metabolites M6 (3,4-dihydroxy-ospemifene), M9 (4-hydroxy-hydro-ospemifene), M11 (side chain hydroxylation), are all very polar in nature, and it is quite probable that at least some of these were responsible for the unknown metabolite peaks in the radiochromatograms. Even if some of these unknowns were disproportionate or human-only metabolites, their relative AUC remains below the critical 10% level set by the regulators (should be measured at steady-state though) and they are not considered to raise safety concerns. Furthermore, regarding the worst case scenario, if all were downstream products of reactive metabolites, they represent about 15% of ospemifene-related material and thus comprise together less than 10 mg of the daily dose of 60 mg. Considering type B and C toxicity, less than 10 mg daily excretion of any metabolite is generally judged safe regardless of whether there are reactive metabolites in the pathway.

Overall, the postulated biotransformation pathways for ospemifene (II, Figure 2) hold quite nicely true after quantitative determination of circulating metabolites. Taking into account the nature of the identified metabolites and the relative abundances of all circulating metabolites compared to ospemifene, additional studies would be warranted only for the main metabolite M1. However, as both M1 and M2 are active metabolites possessing parent-like SERM activities (Unkila *et al.* 2013), additional studies were considered for both metabolites.

### *5.1.4 Interspecies comparison of ospemifene main metabolites (V)*

The interspecies comparison of ospemifene metabolism between human and animal species used in toxicological studies concentrated on the most important metabolites M1 and M2, 4- and 4'-hydroxyospemifene.

Incubations of ospemifene with liver homogenates of human, rat, dog and monkey produced significant interspecies differences considering M1 and M2 as described in V, Figure 1. Most notably, rat produced no 4'-hydroxyospemifene

(M2) and dog produced no 4-hydroxyospemifene (M1) *in vitro,* indicating that both animals might be insufficient alone to study the pharmacology and toxicology of ospemifene. Especially dog would be a poor choice due to complete lack of the main human *in vivo* metabolite M1 and very low abundance of the other pharmacologically active metabolite M2. In contrast to rat and dog, monkey might prove to be excellent choice as both active metabolites were produced *in vitro* in high abundances. All the species produced also additional major and minor metabolites, especially glucuronides and dihydroxylations, or other oxidations, but they were not further investigated.

The semiquantitave *in vivo* abundances of ospemifene M1 and M2 metabolites and preliminary LC/MS identification of other metabolites are presented in V, Table 1. The studied samples were taken at steady-state from human, mouse, rat, dog and monkey administered variable dose levels. Most abundant *in vivo* metabolites in animals were 4- and 4'-hydroxylation (M1 and M2), carboxylic acid (M3) and side chain hydroxylation (M11). Additionally, glucuronides (especially hydroxyl-glucuronides) and quinones were detected at high levels. The studied human samples (after 60 mg daily dosing) contained only M1 and M2. Interspecies differences were again significant concerning the production of active metabolites M1 and M2, but due to semiquantitave analytical method and tremendous difference in the administered dose comparison between the species was not meaningful. Nevertheless, the main biotransformation pathways seem to be the same in all studied species, but the major route might differ from species to species. There is also a good correlation to *in vitro* comparison regarding the formation of M1 and M2 (V, Figure 1 vs. Table 1). Even though mouse was not included in the *in vitro* part of this study, it should be noted that in another (unpublished) study with ospemifene and isolated mouse hepatocytes both M1 and M2 were detected in high amounts. Based on these results, the most human-like species are mouse and monkey considering the *in vivo* (and *in vitro*) production of active metabolites M1 and M2.

### *5.1.5 Evaluation of human vs. animal exposure (V)*

The evaluation of animal exposure of human metabolites covers only the most important metabolites M1 and M2, 4- and 4'-hydroxyospemifene.

Regulatory agencies demand proof that active metabolites have had equal or greater than human exposure in at least one animal species used in toxicological testing. Such exposure data for ospemifene and active metabolites M1 and M2 is

available as steady state AUC from human (therapeutic dose) and four animal species (highest dose level in mouse, rat, dog and monkey) in V, Table 2. In all species, the AUC of M1 is significantly greater than the AUC of M2, but the interspecies variation is significant. All species produce quite similar levels of M2 compared to ospemifene, but very different levels of M1. The human exposure of M2 was clearly exceeded by all other species except rat (close to equal) and dog, which did not produce that metabolite at all. The human exposure of the most important metabolite M1 was by far exceeded by all other species except dog. Concerning the results being the poorest in dog, it is very much in line with *in vitro* results, even *in vitro* M1 was missing and M2 was minute. The exposures of both active metabolites were simultaneously exceeded in mouse and monkey. The exposure was especially sufficient in monkey, which produced the highest levels of both metabolites at a relatively low dose level as predicted by the *in vitro* results.

Considering the observed interspecies exposure, especially in monkey and mouse, it may be presumed that the contribution of active metabolites to the overall toxicity of ospemifene has been well taken into account during the toxicological program.

## 5.2 CYP-related drug interactions of ospemifene

The interaction potential of ospemifene was studied *in vitro* and confirmed *in vivo*. First, it was identified which CYP enzymes were responsible for the production of the main oxidative metabolites 4- and 4'-hydrosxyospemifene, M1 and M2. There were two approaches: utilization of recombinant CYP enzymes one by one to produce these metabolites, and inhibition of CYP enzymes one by one to reduce the production of the metabolites in human liver microsomes. Secondly, the roles of suspected CYP enzymes were confirmed in clinical studies with ospemifene and selected inhibitors. Thirdly, the inhibition potential of ospemifene towards CYP enzymes was studied in human liver microsomes and the induction potential was studied in isolated human hepatocytes. Finally, the CYP inhibition and induction potential (or rather lack of them) was confirmed in clinical studies with ospemifene and selected concomitant drugs. Some of the *in vitro* studies were also performed with ospemifene metabolites M1 and M2.

### 5.2.1 Recombinant CYP enzymes and formation of M1 and M2 (II)

The participation of different human CYP enzymes in the formation of 4- and 4'-hydroxyospemifene (M1 and M2) was first studied using commercially available insect cell microsomes containing cDNA-expressed enzymes. The results are presented in II, Figure 4. The extrapolation of the production of ospemifene hydroxylations from recombinant enzymes to microsomes takes into account the average content of each CYP enzyme in microsomes according to Rostami-Hodjegan & Tucker (2007). The results are surprisingly different for the metabolites. For M2 (4'-hydroxyospemifene) CYP3A4 seems to be practically the only enzyme involved in the reaction, whereas for M1 (4-hydroxyospemifene), CYP2C19 was the most active enzyme, followed by CYP2B6, CYP2C9 and CYP 3A4 (in descending order).

Putting together the CYP assignment experiments with recombinant enzymes and taking into account the relative abundances of the main metabolites, it can be anticipated that no CYP enzyme will be in bottleneck position. In fact, several CYP enzymes, namely CYP2C9, CYP2C19, CYP2B6, and CYP3A4, are likely to take part in the formation the metabolites M1 and M2. It should be stressed, however, that the use of a highly isolated system (recombinant enzymes) with a single ospemifene concentration does not take into account the *in vivo* interplay of other metabolizing enzymes and transporters, and can only give semiquantitative information at best. Tentatively, the participation of the indicated enzymes in ospemifene metabolism was suggested.

### 5.2.2 CYP-specific inhibitors and in vitro formation of M1 and M2 (IV)

The *in vitro* study about the participation of different human CYP enzymes in the formation of 4- and 4'-hydroxyospemifene (M1 and M2) continued with microsomal incubations using CYP-selective inhibitors to disable CYP enzymes one by one and measuring the effect in the production of the metabolites. The results are presented in IV, Table 1. The principal catalyst for 4'-hydroxyospemifene (M2) formation was found to be CYP3A4, as predicted by the use of recombinant CYP enzymes, with CYP2C19 and CYP2B6 possessing minor catalytic potential. Concerning the formation of 4-hydroxyospemifene (M1), it appears to be catalyzed by many CYP enzymes. CYP2C9 was found to be the principal catalyst, with CYP2C19, CYP2B6 and CYP3A4 having also important catalytic activity, also as predicted by the use of recombinant enzymes.

The fact that both approaches point out the same CYP enzymes in the same relative order is quite convincing evidence about their involvement in the production of the main metabolites (M1 and M2) of ospemifene.

The contributions of these CYP enzymes to ospemifene hydroxylations were further examined in human microsome incubations with ospemifene and the pinpointed CYP-selective inhibitors itraconazole (CYP3A4), sulfaphenazole (CYP2C9), fluconazole (CYP2C19) and thioTEPA (CYP2B6). At the lowest ospemifene concentration of 0.5 μM, which is close to a therapeutic *in vivo* concentration, inhibition of ospemifene 4-hydroxylation was 49%, 22%, 23% and 0% with itraconazole, fluconazole, sulfaphenazole and thio-TEPA, respectively. The respective inhibition of ospemifene 4'-hydroxylation was 56%, 31%, 7% and 4%. None of the investigated CYP inhibitors caused complete inhibition of ospemifene metabolism *in vitro*. At clinically relevant concentration, the inhibition of CYP2B6 was practically negligible by thioTEPA. Based on this comparison and the IC50 values for CYP-selective inhibitors (IV, Figure 1), no individual CYP predominates in the formation of the main metabolites M1 and M2, and the most important enzymes are likely to be CYP3A4, CYP2C9 and CYP2C19, in this order of importance.

Additionally, as the main metabolites of ospemifene (M1 and M2) are suspected to undergo further CYP-mediated metabolism, the CYP-specific inhibitors were used to study their impact on the formation of metabolism of M1 and M2. The results suggest that CYP3A4 was the only significant *in vitro* contributor to the further metabolism of 4-hydroxyospemifene and 4'-hydroxyospemifene in human liver microsomes. This further enhances the suspicion about the most important role of CYP3A4 in the oxidative metabolism of ospemifene.

In all cases, the inhibition of ospemifene metabolism was incomplete, even at the highest concentrations of the inhibitors examined in these studies. The *in vitro* studies suggest that no single CYP isoform is critical for ospemifene metabolism and several CYP enzymes contribute to the metabolic clearance of ospemifene.

### *5.2.3 Clinical studies with ospemifene as a victim (IV)*

Two Phase I clinical trials in postmenopausal women were conducted to confirm the role of CYP3A4, CYP2C9 and CYP2C19 on the metabolism and pharmacokinetics of ospemifene, as the *in vitro* interaction studies pointed out their potential importance in the clearance of ospemifene.

In the first trial the pharmacokinetics of ospemifene was examined in the presence of rifampicin (strong CYP3A and CYP2C9 inducer) or ketoconazole (modest CYP3A4 inhibitor) and compared with the PK of ospemifene alone. The pharmacokinetic parameters of ospemifene and 4-hydroxyospemifene were determined and are given in IV, Table 2 and Figure 1. The mean exposure to ospemifene was approximately 60% lower after rifampicin and 40% higher after ketoconazole compared to ospemifene alone. Similarly, maximum serum concentrations (Cmax) decreased significantly with rifampicin and increased with ketoconazole. The pharmacokinetic results for ospemifene were as expected based on *in vitro* investigations.

The serum concentrations and pharmacokinetics of 4-hydroxyospemifene (M1) were affected by rifampicin and ketoconazole pretreatment in a manner similar to those of ospemifene (IV, Table 2 and Figure 1). This is somewhat unexpected at first sight, as one would expect the concentrations of a metabolite to increase after induction and decrease after inhibition of metabolizing CYP enzymes. Based on *in vitro* results, CYP3A4 is practically the only CYP enzyme catalyzing the further metabolism of 4-hydroxyospemifene. Thus, after strong induction of CYP3A4 by rifampicin, not only does the formation of M1 from ospemifene increase, but also the elimination of M1 increases. As the formation of M1 is dependent on several CYPs, it is likely that the rate of elimination of M1 is affected much more than the formation. Likewise, the inhibition of CYP3A4 by ketoconazole probably decreased the formation of M1 from ospemifene less than the elimination of M1 causing the overall increase in the concentrations of M1.

The second trial examined the effects of fluconazole (potent CYP3A, CYP2C9 and CYP2C19 inhibitor) and omeprazole (potent CYP2C19 inhibitor) on ospemifene pharmacokinetics. The PK parameters of ospemifene, 4-hydroxyospemifene and also 4'-hydroxyospemifene were determined and are given in IV, Table 3 and Figure 2. The concentrations of ospemifene increased after fluconazole pretreatment and were essentially unaffected by concomitant omeprazole treatment. The coadministration of fluconazole increased the mean exposure to ospemifene by approximately 170%, whereas the coadministration of omeprazole increased the mean exposure to ospemifene only by approximately 15%. Cmax was 70% higher after fluconazole administration and 20% higher after omeprazole administration than after ospemifene alone. Fluconazole extended the half-life of ospemifene by about 70% while omeprazole had no influence. Fluconazole increased the AUC of ospemifene, as would be expected due to the inhibition of CYP3A, CYP2C9 and CYP2C19. The relatively small

impact of omeprazole to the AUC of ospemifene is expected based on the *in vitro* finding that CYP2C19 is a less important enzyme for the metabolism of ospemifene than CYP3A4 and CYP2C9 are.

The concentrations and pharmacokinetic parameters of the metabolites were affected to a lesser degree by fluconazole than those of the parent compound. One would have expected a fall in the concentrations and AUC of M1 (4-hydroxyospemifene), as all the important CYP enzymes involved in the formation from ospemifene were inhibited by fluconazole. It is probable that the inhibition of also the only important CYP enzyme in elimination of M1 balanced the situation, together with the fact that the inhibition of formation must have been incomplete. Similarly, the elimination of M2 (4'-hydroxyospemifene) was inhibited more by fluconazole than its formation, hence the increased AUC. Omeprazole treatment had no significant effects on the concentrations or pharmacokinetics of ospemifene metabolites.

These *in vivo* PK results are consistent with the *in vitro* findings that CYP3A4 is the most important CYP isoform for the metabolism of ospemifene, followed by CYP2C9 and CYP2C19. Additionally, it is likely that other isoforms contribute to ospemifene metabolism, but to a lesser extent. Furthermore, the clinical use of ospemifene with potent CYP3A and CYP2C9 inducers (especially rifampicin) may significantly decrease the exposure to ospemifene. Ospemifene should be used cautiously in the presence of CYP3A4 inhibitors (like ketoconazole) and, especially, its use with the potent CYP3A, CYP2C9 and CYP2C19 inhibitor fluconazole should be avoided.

### *5.2.4 CYP inhibition potency of ospemifene (III)*

Comparison of the inhibitory potencies of ospemifene, 4-hydroxyospemifene, and 4'-hydroxyospemifene as measured using the current N-in-one assay in human liver microsomes *in vitro* is presented in III, Table 1. Ospemifene was a weak inhibitor of CYP2B6 ($IC_{50}$, 7.8 µM) and CYP2C9 ($IC_{50}$, 10 µM) and showed also some inhibiting potency towards CYP2C19, CYP2C8, CYP2D6 and CYP3A4 ($IC_{50}$ 23–49 µM. At steady state, the mean $C_{max}$ for ospemifene was found to be 785 ng/mL, corresponding to roughly 2 µM (Koskimies *et al.* 2013). Further studies indicated that ospemifene inhibition was competitive for CYP2B6 and CYP2D6 and likely to be competitive for CYP2C8. Ospemifene inhibition of CYP2C9 was competitive up to 10 µM, but a noncompetitive component

emerged at higher concentrations. The inhibition of CYP2C19 was noncompetitive.

Metabolite M1, 4-hydroxyospemifene, potently inhibited CYP2C9 activity, $IC_{50}$ being 1.1 μM. Inhibition of CYP2C19 activity was approximately an order of magnitude less potent. $IC_{50}$ values for all other CYP-mediated enzyme activities were >25 μM, well above the clinical $C_{max}$ of 4-hydroxyospemifene, approximately 0.3 μM, after repeated oral 60-mg dosing. Metabolite M2, 4'-hydroxyospemifene, inhibited CYP2C8 activity with the highest potency ($IC_{50}$, 7 μM). CYP2C9 and CYP2B6 were also inhibited by 4'-hydroxyospemifene. $IC_{50}$ values for all other CYP-mediated reactions were >50 μM. The clinical $C_{max}$ of 4'-hydroxyospemifene is approximately 0.1 μM for repeated oral 60-mg dosing.

Taken together, ospemifene and its main metabolites M1 and M2 (4-hydroxyospemifene and 4'-hydroxyospemifene) weakly inhibited a number of CYPs (CYP2B6, CYP2C9, CYP2C19, CYP2C8, and CYP2D6) in human liver microsomal preparations. However, at clinically relevant concentrations only CYP2C9 activity was inhibited by the main metabolite 4-hydroxyospemifene ($IC_{50}$ 1.1 μM). Taking into account the clinical concentrations of M1 ($C_{max}$ 0.3 μM), the clinical interactions due to inhibition of CYP2C9 are likely to be minor. Additionally, time-dependent inhibition was not observed at clinically relevant concentrations.

### *5.2.5 CYP induction potency of ospemifene (III)*

In isolated human hepatocytes, incubation with ospemifene (0.2, 2, and 20 μM) for 48 h did not cause relevant changes in CYP1A2, CYP2C9, or CYP2C19 activity. The highest concentration (20 μM) of ospemifene was associated with approximately two-fold induction of CYP2B6 and CYP3A4 model activities. Three reference CYP inducers (omeprazole, phenobarbital, or rifampicin) induced approximately 1.3- to 41.6-fold increases in the respective CYP enzyme model activities, as expected. Thus, the induction potency of ospemifene towards CYP enzymes was negligible and further studies were not relevant.

### *5.2.6 Clinical studies with ospemifene as a perpetrator (III)*

To confirm the *in vivo* effects of ospemifene on CYP-mediated metabolism, three Phase I pharmacokinetic studies with postmenopausal women were conducted

using recommended probe drugs for CYP2C9 (warfarin), CYP2B6 (bupropion) and CYP2C19 and CYP3A4 (omeprazole) (Pelkonen *et al.* 2008a, EMA 2012).

In the first study, the effect of ospemifene on warfarin pharmacokinetics was studied. The mean plasma concentration curves for S-warfarin (formation catalyzed by CYP2C9) and other PK parameters are presented III, Figure 1 and Table 2, respectively. All results were similar with and without ospemifene, indicating that ospemifene did not interfere with warfarin metabolism. Despite the potency of ospemifene and the relatively high potency of the major metabolite 4-hydroxyospemifene to inhibit CYP2C9 in human liver microsomes *in vitro*, the data did not provide clinical evidence of inhibiting CYP2C9-mediated metabolism.

In the second study, the effect of ospemifene on bupropion pharmacokinetics was studied. The mean plasma concentration curves for bupropion and hydroxybupropion (formation catalyzed by CYP2B6), and other PK parameters are presented III, Figure 2 and Table 2, respectively. All results were similar with and without ospemifene, indicating that ospemifene did not interfere with bupropion metabolism. Despite the potency of ospemifene to inhibit CYP2B6 in human liver microsomes *in vitro*, there was no evidence that ospemifene would affect CYP2B6-mediated drug metabolism in clinical use.

In the third study, the effect of ospemifene on omeprazole pharmacokinetics was studied. The mean plasma concentration curves for omeprazole, 5-hydroxyomeprazole (formation catalyzed by CYP2C19) and omeprazole sulfone (formation catalyzed by CYP3A4) are presented III, Figure 3, and bioequivalence statistics in III, Table 3. Due to high inter-individual variability, bioequivalence could not be concluded at 3 h plasma concentrations. However, bioequivalence could clearly be concluded for the full AUC. These results indicated absence of a pharmacokinetic interaction with and without ospemifene pretreatment. Despite the weak *in vitro* potency of ospemifene and its hydroxylated metabolites with CYP2C19- and CYP3A4-mediated drug metabolism, the clinical study results indicated no effect of ospemifene on most pharmacokinetic parameters related to CYP2C19 and CYP3A4.

Results from these studies indicate low potential for ospemifene to alter the metabolism or pharmacokinetics of drugs that are CYP substrates. There were no clinically relevant changes in pharmacokinetic parameters for warfarin, bupropion, or omeprazole. The results obtained in the preclinical *in vitro* assays were judged to demonstrate a relatively weak inhibitory potency of ospemifene, and indeed, this expectation was confirmed by targeted clinical studies.

# 6 Conclusions

The metabolic properties and drug interaction potential of ospemifene were examined in the present study as an integral part of its drug development program. Practically all of the *in vitro* study data were included in the marketing authorization application of ospemifene as part of the required scientific package. The main findings were:

- Human *in vitro* and *in vivo* metabolites of ospemifene were identified and principal metabolic pathways were proposed. The main metabolic pathway involved initial 4-hydroxylation and the minor pathways began with other aromatic or aliphatic oxidations or with glucuronidation of the parent.
- The important human circulating metabolites were 4-hydroxyospemifene (M1) and 4'-hydroxyospemifene (M2), M1 being the principal metabolite.
- The most human-like animal species were mouse and monkey in the production of the main metabolites M1 and M2.
- The nonclinical animal exposure of the main ospemifene metabolites has been very sufficient to cover the human dose.
- CYP3A4, CYP2C9 and CYP2C19 were found to be the major enzymes to catalyze the oxidative metabolism of ospemifene. CYP3A4 was the most important, but not critical for the ospemifene elimination.
- Clinical ospemifene exposure is likely to notably be affected by strong 3A4 inhibitors or inducers when taken concomitantly.
- The CYP inhibition potency of ospemifene was found to be low at clinically relevant concentrations.
- The CYP induction potency of ospemifene was found to be negligible.
- Ospemifene can be expected to be a metabolically safe drug based on the following factors: no human metabolic liabilities including downstream products of reactive metabolites, no time-dependent CYP inhibition, no CYP interaction potential as perpetrator and quite low daily dose of 60 mg. Being victim to CYP3A4 interactions is the only safety risk related to metabolism, which can lead to elevated or decreased ospemifene exposures, and thus to adverse events (elevated exposure) or therapeutic failure (decreased exposure) of a postmenopausal condition, which is not life-threatening.

– *In vitro – in vivo* extrapolation was highly valid in the ospemifene case. Generally, *in vitro* metabolic profiles were predictive of *in vivo* profiles in humans and the animal species studied, and human *in vitro* CYP interaction potential was very predictive of *in vivo* pharmacokinetic situation.

# References


Aller SG, Yu J, Ward A, Weng Y, Chittaboina S, Zhuo R, Harrell PM, Trinh YT, Zhang Q, Urbatsch IL & Chang G (2009) Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. Science 323(5922): 1718–1722.

Anderson S, Luffer-Atlas D & Knadler MP (2009) Predicting circulating human metabolites: how good are we? Chem Res Toxicol 22(2): 243–256.

Antherieu S, Chesne C, Li R, Camus S, Lahoz A, Picazo L, Turpeinen M, Tolonen A, Uusitalo J, Guguen-Guillouzo C & Guillouzo A (2010) Stable expression, activity, and inducibility of cytochromes P450 in differentiated HepaRG cells. Drug Metab Dispos 38(3): 516–525.

Argoti D, Liang L, Conteh A, Chen L, Bershas D, Yu CP, Vouros P & Yang E (2005) Cyanide trapping of iminium ion reactive intermediates followed by detection and structure identification using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Chem Res Toxicol 18(10): 1537–1544.

Arrowsmith J (2011a) Trial watch: Phase II failures: 2008–2010. Nat Rev Drug Discov 10(5): 328–329.

Arrowsmith J (2011b) Trial watch: phase III and submission failures: 2007–2010. Nat Rev Drug Discov 10(2): 87.

Arrowsmith J & Miller P (2013) Trial watch: phase II and phase III attrition rates 2011–2012. Nat Rev Drug Discov 12(8): 569.

Bailey MJ & Dickinson RG (2003) Acyl glucuronide reactivity in perspective: biological consequences. Chem Biol Interact 145(2): 117–137.

Baillie TA (2006) Future of toxicology-metabolic activation and drug design: challenges and opportunities in chemical toxicology. Chem Res Toxicol 19(7): 889–893.

Baillie TA (2008) Metabolism and toxicity of drugs. Two decades of progress in industrial drug metabolism. Chem Res Toxicol 21(1): 129–137.

Baillie TA (2009) Approaches to the assessment of stable and chemically reactive drug metabolites in early clinical trials. Chem Res Toxicol 22(2): 263–266.

Baillie TA, Cayen MN, Fouda H, Gerson RJ, Green JD, Grossman SJ, Klunk LJ, LeBlanc B, Perkins DG & Shipley LA (2002) Drug metabolites in safety testing. Toxicol Appl Pharmacol 182(3): 188–196.

Baillie TA, Cayen MN, Fouda H, Gerson RJ, Green JD, Grossman SJ, Klunk LJ, LeBlanc B, Perkins DG & Shipley LA (2003) Reply. Toxicol Appl Pharmacol 190: 93–94.

Ballard P, Brassil P, Bui KH, Dolgos H, Petersson C, Tunek A & Webborn PJ (2012) The right compound in the right assay at the right time: an integrated discovery DMPK strategy. Drug Metab Rev 44(3): 224–252.

Barbara JE, Kazmi F, Muranjan S, Toren PC & Parkinson A (2012) High-resolution mass spectrometry elucidates metabonate (false metabolite) formation from alkylamine drugs during in vitro metabolite profiling. Drug Metab Dispos 40(10): 1966–1975.

Bass AS, Cartwright ME, Mahon C, Morrison R, Snyder R, McNamara P, Bradley P, Zhou YY & Hunter J (2009) Exploratory drug safety: a discovery strategy to reduce attrition in development. J Pharmacol Toxicol Methods 60(1): 69–78.

Bateman KP, Castro-Perez J, Wrona M, Shockcor JP, Yu K, Oballa R & Nicoll-Griffith DA (2007) MSE with mass defect filtering for in vitro and in vivo metabolite identification. Rapid Commun Mass Spectrom 21(9): 1485–1496.

Bateman KP, Kellmann M, Muenster H, Papp R & Taylor L (2009) Quantitative-qualitative data acquisition using a benchtop Orbitrap mass spectrometer. J Am Soc Mass Spectrom 20(8): 1441–1450.

Bateman TJ, Reddy VG, Kakuni M, Morikawa Y & Kumar S (2014) Application of chimeric mice with humanized liver for study of human-specific drug metabolism. Drug Metab Dispos 42(6): 1055–1065.

Bauman JN, Kelly JM, Tripathy S, Zhao SX, Lam WW, Kalgutkar AS & Obach RS (2009) Can in vitro metabolism-dependent covalent binding data distinguish hepatotoxic from nonhepatotoxic drugs? An analysis using human hepatocytes and liver S-9 fraction. Chem Res Toxicol 22(2): 332–340.

Benet LZ (2010) Predicting drug disposition via application of a Biopharmaceutics Drug Disposition Classification System. Basic Clin Pharmacol Toxicol 106(3): 162–167.

Bergstrom CA, Holm R, Jorgensen SA, Andersson SB, Artursson P, Beato S, Borde A, Box K, Brewster M, Dressman J, Feng KI, Halbert G, Kostewicz E, McAllister M, Muenster U, Thinnes J, Taylor R & Mullertz A (2014) Early pharmaceutical profiling to predict oral drug absorption: current status and unmet needs. Eur J Pharm Sci 57: 173–199.

Berthou F & Dreano Y (1993) High-performance liquid chromatographic analysis of tamoxifen, toremifene and their major human metabolites. J Chromatogr 616(1): 117–127.

Blech S & Laux R (2013) Resolving the microcosmos of complex samples: UPLC/travelling wave ion mobility separation high resolution mass spectrometry for the analysis of in vivo drug metabolism studies. int J, Ion Mobil Spec 16: 5–17.

Bode C (2010) The nasty surprise of a complex drug-drug interaction. Drug Discov Today 15(9–10): 391–395.

Bonn B, Leandersson C, Fontaine F & Zamora I (2010) Enhanced metabolite identification with MS(E) and a semi-automated software for structural elucidation. Rapid Commun Mass Spectrom 24(21): 3127–3138.

Bouzom F, Ball K, Perdaems N & Walther B (2012) Physiologically based pharmacokinetic (PBPK) modelling tools: how to fit with our needs? Biopharm Drug Dispos 33(2): 55–71.

Boyd RA & Lalonde RL (2007) Nontraditional approaches to first-in-human studies to increase efficiency of drug development: will microdose studies make a significant impact? Clin Pharmacol Ther 81(1): 24–26.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.

Brandon EF, Raap CD, Meijerman I, Beijnen JH & Schellens JH (2003) An update on in vitro test methods in human hepatic drug biotransformation research: pros and cons. Toxicol Appl Pharmacol 189(3): 233–246.

Brink A, Fontaine F, Marschmann M, Steinhuber B, Cece EN, Zamora I & Pahler A (2014) Post-acquisition analysis of untargeted accurate mass quadrupole time-of-flight MS(E) data for multiple collision-induced neutral losses and fragment ions of glutathione conjugates. Rapid Commun Mass Spectrom 28(24): 2695–2703.

Brouwer KL, Keppler D, Hoffmaster KA, Bow DA, Cheng Y, Lai Y, Palm JE, Stieger B, Evers R & International Transporter Consortium (2013) In vitro methods to support transporter evaluation in drug discovery and development. Clin Pharmacol Ther 94(1): 95–112.

Brown HS, Griffin M & Houston JB (2007) Evaluation of cryopreserved human hepatocytes as an alternative in vitro system to microsomes for the prediction of metabolic clearance. Drug Metab Dispos 35(2): 293–301.

Brown K, Tompkins EM & White IN (2006) Applications of accelerator mass spectrometry for pharmacological and toxicological research. Mass Spectrom Rev 25(1): 127–145.

Bryson S, Cornelisson K & Anttila M (2005) Absorption, metabolism and excretion of (3H)-ospemifene following a single oral dose to postmenopausal women. Clin Pharmacol Ther 77(2): P82.

Buscher B, Laakso S, Mascher H, Pusecker K, Doig M, Dillen L, Wagner-Redeker W, Pfeifer T, Delrat P & Timmerman P (2014) Bioanalysis for plasma protein binding studies in drug discovery and drug development: views and recommendations of the European Bioanalysis Forum. Bioanalysis 6(5): 673–682.

Calcoen D, Elias L & Yu X (2015) What does it take to produce a breakthrough drug? Nat Rev Drug Discov 14(3): 161–162.

Callegari E, Kalgutkar AS, Leung L, Obach RS, Plowchalk DR & Tse S (2013) Drug metabolites as cytochrome p450 inhibitors: a retrospective analysis and proposed algorithm for evaluation of the pharmacokinetic interaction potential of metabolites in drug discovery and development. Drug Metab Dispos 41(12): 2047–2055.

Castro-Perez J, Plumb R, Granger JH, Beattie I, Joncour K & Wright A (2005) Increasing throughput and information content for in vitro drug metabolism experiments using ultra-performance liquid chromatography coupled to a quadrupole time-of-flight mass spectrometer. Rapid Commun Mass Spectrom 19(6): 843–848.

Chan TS, Yu H, Moore A, Khetani SR & Tweedie D (2013) Meeting the challenge of predicting hepatic clearance of compounds slowly metabolized by cytochrome P450 using a novel hepatocyte model, HepatoPac. Drug Metab Dispos 41(12): 2024–2032.

Chen M, Borlak J & Tong W (2013) High lipophilicity and high daily dose of oral medications are associated with significant risk for drug-induced liver injury. Hepatology 58(1): 388–396.

Chen Y, Jin JY, Mukadam S, Malhi V & Kenny JR (2012) Application of IVIVE and PBPK modeling in prospective prediction of clinical pharmacokinetics: strategy and approach during the drug discovery phase with four case studies. Biopharm Drug Dispos 33(2): 85–98.

Chen Y, Liu L, Monshouwer M & Fretland AJ (2011) Determination of time-dependent inactivation of CYP3A4 in cryopreserved human hepatocytes and assessment of human drug-drug interactions. Drug Metab Dispos 39(11): 2085–2092.

Choi JM, Oh SJ, Lee JY, Jeon JS, Ryu CS, Kim YM, Lee K & Kim SK (2015) Prediction of Drug-Induced Liver Injury in HepG2 Cells Cultured with Human Liver Microsomes. Chem Res Toxicol 28(5): 872–885.

Choy YB & Prausnitz MR (2011) The rule of five for non-oral routes of drug delivery: ophthalmic, inhalation and transdermal. Pharm Res 28(5): 943–948.

Comezoglu SN, Ly VT, Zhang D, Humphreys WG, Bonacorsi SJ, Everett DW, Cohen MB, Gan J, Beumer JH, Beijnen JH, Schellens HM & Lappin G (2009) Biotransformation profiling of [(14)C]ixabepilone in human plasma, urine and feces samples using accelerator mass spectrometry (AMS). Drug Metab Pharmacokinet 24(6): 511–522.

Cook D, Brown D, Alexander R, March R, Morgan P, Satterthwaite G & Pangalos MN (2014) Lessons learned from the fate of AstraZeneca's drug pipeline: a five-dimensional framework. Nat Rev Drug Discov 13(6): 419–431.

Cui Y, Zong H, Yan H, Li N & Zhang Y (2014) The efficacy and safety of ospemifene in treating dyspareunia associated with postmenopausal vulvar and vaginal atrophy: a systematic review and meta-analysis. J Sex Med 11(2): 487–497.

Cumming JG, Davis AM, Muresan S, Haeberlein M & Chen H (2013) Chemical predictive modelling to improve compound quality. Nat Rev Drug Discov 12(12): 948–962.

Cuyckens F, Balcaen LI, De Wolf K, De Samber B, Van Looveren C, Hurkmans R & Vanhaecke F (2008) Use of the bromine isotope ratio in HPLC-ICP-MS and HPLC-ESI-MS analysis of a new drug in development. Anal Bioanal Chem 390(7): 1717–1729.

Cuyckens F, Hurkmans R, Castro-Perez JM, Leclercq L & Mortishire-Smith RJ (2009) Extracting metabolite ions out of a matrix background by combined mass defect, neutral loss and isotope filtration. Rapid Commun Mass Spectrom 23(2): 327–332.

Dahal UP, Jones JP, Davis JA & Rock DA (2011) Small molecule quantification by liquid chromatography-mass spectrometry for metabolites of drugs and drug candidates. Drug Metab Dispos 39(12): 2355–2360.

Dalvie D, Obach RS, Kang P, Prakash C, Loi CM, Hurst S, Nedderman A, Goulet L, Smith E, Bu HZ & Smith DA (2009) Assessment of three human in vitro systems in the generation of major human excretory and circulating metabolites. Chem Res Toxicol 22(2): 357–368.

Davis-Bruno KL & Atrakchi A (2006) A regulatory perspective on issues and approaches in characterizing human metabolites. Chem Res Toxicol 19(12): 1561–1563.

Day SH, Mao A, White R, Schulz-Utermoehl T, Miller R & Beconi MG (2005) A semi-automated method for measuring the potential for protein covalent binding in drug discovery. J Pharmacol Toxicol Methods 52(2): 278–285.

de Vlieger JS, Kolkman AJ, Ampt KA, Commandeur JN, Vermeulen NP, Kool J, Wijmenga SS, Niessen WM, Irth H & Honing M (2010) Determination and identification of estrogenic compounds generated with biosynthetic enzymes using hyphenated screening assays, high resolution mass spectrometry and off-line NMR. J Chromatogr B Analyt Technol Biomed Life Sci 878(7–8): 667–674.

Dear GJ, Roberts AD, Beaumont C & North SE (2008) Evaluation of preparative high performance liquid chromatography and cryoprobe-nuclear magnetic resonance spectroscopy for the early quantitative estimation of drug metabolites in human plasma. J Chromatogr B Analyt Technol Biomed Life Sci 876(2): 182–190.

DeGregorio MW, Wurz GT, Taras TL, Erkkola RU, Halonen KH & Huupponen RK (2000) Pharmacokinetics of (deaminohydroxy)toremifene in humans: a new, selective estrogen-receptor modulator. Eur J Clin Pharmacol 56(6–7): 469–475.

Di L, Atkinson K, Orozco CC, Funk C, Zhang H, McDonald TS, Tan B, Lin J, Chang C & Obach RS (2013b) In vitro-in vivo correlation for low-clearance compounds using hepatocyte relay method. Drug Metab Dispos 41(12): 2018–2023.

Di L, Feng B, Goosen TC, Lai Y, Steyn SJ, Varma MV & Obach RS (2013a) A perspective on the prediction of drug pharmacokinetics and disposition in drug research and development. Drug Metab Dispos 41(12): 1975–1993.

Di L, Trapa P, Obach RS, Atkinson K, Bi YA, Wolford AC, Tan B, McDonald TS, Lai Y & Tremaine LM (2012) A novel relay method for determining low-clearance values. Drug Metab Dispos 40(9): 1860–1865.

DiMasi JA, Feldman L, Seckler A & Wilson A (2010) Trends in risks associated with new drug development: success rates for investigational drugs. Clin Pharmacol Ther 87(3): 272–277.

Dobo KL, Obach RS, Luffer-Atlas D & Bercu JP (2009) A strategy for the risk assessment of human genotoxic metabolites. Chem Res Toxicol 22(2): 348–356.

Dueker SR, Vuong le T, Lohstroh PN, Giacomo JA & Vogel JS (2011) Quantifying exploratory low dose compounds in humans with AMS. Adv Drug Deliv Rev 63(7): 518–531.

Ebner T, Ishiguro N & Taub ME (2015) The Use of Transporter Probe Drug Cocktails for the Assessment of Transporter-Based Drug-Drug Interactions in a Clinical Setting-Proposal of a Four Component Transporter Cocktail. J Pharm Sci : 10.1002/jps.24489 [doi].

Elkinson S & Yang LP (2013) Ospemifene: first global approval. Drugs 73(6): 605–612.

Enoch SJ & Cronin MT (2010) A review of the electrophilic reaction chemistry involved in covalent DNA binding. Crit Rev Toxicol 40(8): 728–748.

Espina R, Yu L, Wang J, Tong Z, Vashishtha S, Talaat R, Scatina J & Mutlib A (2009) Nuclear magnetic resonance spectroscopy as a quantitative tool to determine the concentrations of biologically produced metabolites: implications in metabolites in safety testing. Chem Res Toxicol 22(2): 299–310.

European Medicine Agency (EMA), Committee for Human Medicinal Products (CHMP) ICH guideline M3(R2) on non-clinical safety studies for the conduct of human clinical trials and marketing authorisation for pharmaceuticals. 2009 (December 2009).

European Medicine Agency (EMA), Committee for Human Medicinal Products (CHMP) (2011) ICH guideline M3 (R2) – questions and answers. (July 2011).

European Medicine Agency (EMA), Committee for Human Medicinal Products (CHMP) (2012) Guideline on the Investigation of Drug Interactions. June 21, 2012.

European Medicines Agency (EMA) (2014) EMA recommends 81 medicines for marketing authorisation in 2013. http://www ema europa eu/ema/index jsp?curl=pages/news_and_events/news/2014/01/news_detail_002006 jsp&mid=WC0b01ac058004d5c1 Accessed 10 Jan 2015.

European Medicines Agency (EMA) (2015a) Annual report 2014. http://www ema europa eu/docs/en_GB/document_library/Annual_report/2015/04/WC500186306 pdf : Accessed 14 May 2015.

European Medicines Agency (EMA) (2015b) Annexes to the annual report of the European Medicines Agency 2014. http://www ema europa eu/docs/en_GB/document_library/Other/2015/04/WC500186305 pdf : Accessed 14 May 2015.

Evans DC, Watt AP, Nicoll-Griffith DA & Baillie TA (2004) Drug-protein adducts: an industry perspective on minimizing the potential for drug bioactivation in drug discovery and development. Chem Res Toxicol 17(1): 3–16.

Fan PW, Zhang F & Bolton JL (2000) 4-Hydroxylated metabolites of the antiestrogens tamoxifen and toremifene are metabolized to unusually stable quinone methides. Chem Res Toxicol 13(1): 45–52.

Fashe MM, Juvonen RO, Petsalo A, Rahnasto-Rilla M, Auriola S, Soininen P, Vepsalainen J & Pasanen M (2014) Identification of a new reactive metabolite of pyrrolizidine alkaloid retrorsine: (3H-pyrrolizin-7-yl)methanol. Chem Res Toxicol 27(11): 1950–1957.

Foster JA, Houston JB & Hallifax D (2011) Comparison of intrinsic clearances in human liver microsomes and suspended hepatocytes from the same donor livers: clearance-dependent relationship and implications for prediction of in vivo clearance. Xenobiotica 41(2): 124–136.

Foti RS, Tyndale RF, Garcia KL, Sweet DH, Nagar S, Sharan S & Rock DA (2015) "Target-Site" Drug Metabolism and Transport. Drug Metab Dispos 43(8): 1156–1168.

Frederick CB & Obach RS (2010) Metabolites in safety testing: "MIST" for the clinical pharmacologist. Clin Pharmacol Ther 87(3): 345–350.

Fura A (2006) Role of pharmacologically active metabolites in drug discovery and development. Drug Discov Today 11(3–4): 133–142.

Galetin A, Gertz M & Houston JB (2010) Contribution of intestinal cytochrome p450-mediated metabolism to drug-drug inhibition and induction interactions. Drug Metab Pharmacokinet 25(1): 28–47.

Gan J, Harper TW, Hsueh MM, Qu Q & Humphreys WG (2005) Dansyl glutathione as a trapping agent for the quantitative estimation and identification of reactive metabolites. Chem Res Toxicol 18(5): 896–903.

Gan J, Ruan Q, He B, Zhu M, Shyu WC & Humphreys WG (2009) In vitro screening of 50 highly prescribed drugs for thiol adduct formation – comparison of potential for drug-induced toxicity and extent of adduct formation. Chem Res Toxicol 22(4): 690–698.

Gao H, Deng S & Obach RS (2010) A simple liquid chromatography-tandem mass spectrometry method to determine relative plasma exposures of drug metabolites across species for metabolite safety assessments. Drug Metab Dispos 38(12): 2147–2156.

Gao H, Jacobs A, White RE, Booth BP & Obach RS (2013) Meeting report: metabolites in safety testing (MIST) symposium-safety assessment of human metabolites: what's REALLY necessary to ascertain exposure coverage in safety tests? AAPS J 15(4): 970–973.

Gao H & Obach RS (2012) A simple liquid chromatography-tandem mass spectrometry method to determine relative plasma exposures of drug metabolites across species for metabolite safety assessments (metabolites in safety testing). II. Application to unstable metabolites. Drug Metab Dispos 40(7): 1290–1296.

Gao L, Li J, Kasserra C, Song Q, Arjomand A, Hesk D & Chowdhury SK (2011) Precision and accuracy in the quantitative analysis of biological samples by accelerator mass spectrometry: application in microdose absolute bioavailability studies. Anal Chem 83(14): 5607–5616.

Gao Y, Shao J, Jiang Z, Chen J, Gu S, Yu S, Zheng K & Jia L (2014) Drug enterohepatic circulation and disposition: constituents of systems pharmacokinetics. Drug Discov Today 19(3): 326–340.

Garner RC (2005) Less is more: the human microdosing concept. Drug Discov Today 10(7): 449–451.

Garofolo F, Savoie N, Bergeron A, Amestoy C, Lefebvre M, Caille L, Furtado M & Menard C (2010) Importance of metabolite testing in regulated bioanalysis. Bioanalysis 2(7): 1171–1174.

Gerin B, Dell'Aiera S, Richert L, Smith S & Chanteux H (2013) Assessment of cytochrome P450 (1A2, 2B6, 2C9 and 3A4) induction in cryopreserved human hepatocytes cultured in 48-well plates using the cocktail strategy. Xenobiotica 43(4): 320–335.

Gertz M, Harrison A, Houston JB & Galetin A (2010) Prediction of human intestinal first-pass metabolism of 25 CYP3A substrates from in vitro clearance and permeability data. Drug Metab Dispos 38(7): 1147–1158.

Gleeson MP (2008) Generation of a set of simple, interpretable ADMET rules of thumb. J Med Chem 51(4): 817–834.

Gleeson MP, Hersey A, Montanari D & Overington J (2011) Probing the links between in vitro potency, ADMET and physicochemical parameters. Nat Rev Drug Discov 10(3): 197–208.

Gomez-Lechon MJ, Castell JV & Donato MT (2007) Hepatocytes – the choice to investigate drug metabolism and toxicity in man: in vitro variability as a reflection of in vivo. Chem Biol Interact 168(1): 30–50.

Gradinaru J, Romand S, Geiser L, Carrupt PA, Spaggiari D & Rudaz S (2015) Inhibition screening method of microsomal UGTs using the cocktail approach. Eur J Pharm Sci 71: 35–45.

Graham RA, Lum BL, Morrison G, Chang I, Jorga K, Dean B, Shin YG, Yue Q, Mulder T, Malhi V, Xie M, Low JA & Hop CE (2011) A single dose mass balance study of the Hedgehog pathway inhibitor vismodegib (GDC-0449) in humans using accelerator mass spectrometry. Drug Metab Dispos 39(8): 1460–1467.

Greene N, Aleo MD, Louise-May S, Price DA & Will Y (2010) Using an in vitro cytotoxicity assay to aid in compound selection for in vivo safety studies. Bioorg Med Chem Lett 20(17): 5308–5312.

Grillo JA, Zhao P, Bullock J, Booth BP, Lu M, Robie-Suh K, Berglund EG, Pang KS, Rahman A, Zhang L, Lesko LJ & Huang SM (2012) Utility of a physiologically-based pharmacokinetic (PBPK) modeling approach to quantitatively predict a complex drug-drug-disease interaction scenario for rivaroxaban during the drug review process: implications for clinical practice. Biopharm Drug Dispos 33(2): 99–110.

Grimm SW, Einolf HJ, Hall SD, He K, Lim HK, Ling KH, Lu C, Nomeir AA, Seibert E, Skordos KW, Tonn GR, Van Horn R, Wang RW, Wong YN, Yang TJ & Obach RS (2009) The conduct of in vitro studies to address time-dependent inhibition of drug-metabolizing enzymes: a perspective of the pharmaceutical research and manufacturers of America. Drug Metab Dispos 37(7): 1355–1370.

Guengerich FP (2006) Safety assessment of stable drug metabolites. Chem Res Toxicol 19(12): 1559–1560.

Guengerich FP (2008) Cytochrome p450 and chemical toxicology. Chem Res Toxicol 21(1): 70–83.

Guengerich FP & MacDonald JS (2007) Applying mechanisms of chemical toxicity to predict drug safety. Chem Res Toxicol 20(3): 344–369.

Guillouzo A, Corlu A, Aninat C, Glaise D, Morel F & Guguen-Guillouzo C (2007) The human hepatoma HepaRG cells: a highly differentiated model for studies of liver metabolism and toxicity of xenobiotics. Chem Biol Interact 168(1): 66–73.

Guiney WJ, Beaumont C & Thomas SR (2010) Use of the entero-test, a novel approach for the noninvasive capture of biliary metabolites in dogs. Drug Metab Dispos 38(5): 851–856.

Gustafsson F, Foster AJ, Sarda S, Bridgland-Taylor MH & Kenna JG (2014) A correlation between the in vitro drug toxicity of drugs to cell lines that express human P450s and their propensity to cause liver injury in humans. Toxicol Sci 137(1): 189–211.

Haglund J, Halldin MM, Brunnstrom A, Eklund G, Kautiainen A, Sandholm A & Iverson SL (2014) Pragmatic approaches to determine the exposures of drug metabolites in preclinical and clinical subjects in the MIST evaluation of the clinical development phase. Chem Res Toxicol 27(4): 601–610.

Hagmann WK (2008) The discovery of taranabant, a selective cannabinoid-1 receptor inverse agonist for the treatment of obesity. Arch Pharm (Weinheim) 341(7): 405–411.

Hallifax D & Houston JB (2006) Binding of drugs to hepatic microsomes: comment and assessment of current prediction methodology with recommendation for improvement. Drug Metab Dispos 34(4): 724–6; author reply 727.

Hallifax D & Houston JB (2012) Evaluation of hepatic clearance prediction using in vitro data: emphasis on fraction unbound in plasma and drug ionisation using a database of 107 drugs. J Pharm Sci 101(8): 2645–2652.

Hamilton RA, Garnett WR & Kline BJ (1981) Determination of mean valproic acid serum level by assay of a single pooled sample. Clin Pharmacol Ther 29(3): 408–413.

Hanzlik RP, Fang J & Koen YM (2009) Filling and mining the reactive metabolite target protein database. Chem Biol Interact 179(1): 38–44.

Hanzlik RP, Koen YM, Theertham B, Dong Y & Fang J (2007) The reactive metabolite target protein database (TPDB)–a web-accessible resource. BMC Bioinformatics 8: 95.

Hastings KL, El-Hage J, Jacobs A, Leighton J, Morse D & Osterberg RE (2003) Drug metabolites in safety testing. Toxicol Appl Pharmacol 190(1): 91–2;author reply 93–4.

Hay M, Thomas DW, Craighead JL, Economides C & Rosenthal J (2014) Clinical development success rates for investigational drugs. Nat Biotechnol 32(1): 40–51.

Hewitt NJ, Lechon MJ, Houston JB, Hallifax D, Brown HS, Maurel P, Kenna JG, Gustavsson L, Lohmann C, Skonberg C, Guillouzo A, Tuschl G, Li AP, LeCluyse E, Groothuis GM & Hengstler JG (2007) Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. Drug Metab Rev 39(1): 159–234.

Hirouchi M, Kusuhara H, Onuki R, Ogilvie BW, Parkinson A & Sugiyama Y (2009) Construction of triple-transfected cells [organic anion-transporting polypeptide (OATP) 1B1/multidrug resistance-associated protein (MRP) 2/MRP3 and OATP1B1/MRP2/MRP4] for analysis of the sinusoidal function of MRP3 and MRP4. Drug Metab Dispos 37(10): 2103–2111.

Ho S (2014) Best practices for discovery bioanalysis: balancing data quality and productivity. Bioanalysis 6(20): 2705–2708.

Hochman J, Tang C & Prueksaritanont T (2015) Drug-drug interactions related to altered absorption and plasma protein binding: theoretical and regulatory considerations, and an industry perspective. J Pharm Sci 104(3): 916–929.

Holmberg AA, Ekdahl A & Weidolf L (2014) Systemic exposure to the metabolites of lesogaberan in humans and animals: a case study of metabolites in safety testing. Drug Metab Dispos 42(6): 1016–1021.

Hop CE, Wang Z, Chen Q & Kwei G (1998) Plasma-pooling methods to increase throughput for in vivo pharmacokinetic screening. J Pharm Sci 87(7): 901–903.

Houston JB (2013) Prediction of human pharmacokinetics in 2013 and beyond. Drug Metab Dispos 41(12): 1973–1974.

Huang SM (2012) PBPK as a tool in regulatory review. Biopharm Drug Dispos 33(2): 51–52.

Hughes JD, Blagg J, Price DA, Bailey S, Decrescenzo GA, Devraj RV, Ellsworth E, Fobian YM, Gibbs ME, Gilles RW, Greene N, Huang E, Krieger-Burke T, Loesel J, Wager T, Whiteley L & Zhang Y (2008) Physiochemical drug properties associated with in vivo toxicological outcomes. Bioorg Med Chem Lett 18(17): 4872–4875.

Humphreys WG (2011) Overview of strategies for addressing BRIs in drug discovery: Impact on optimization and design. Chem Biol Interact 192(1–2): 56–59.

Humphreys WG & Unger SE (2006) Safety assessment of drug metabolites: Characterization of chemically stable metabolites. Chem Res Toxicol 19(12): 1564–1569.

International Transporter Consortium, Giacomini KM, Huang SM, Tweedie DJ, Benet LZ, Brouwer KL, Chu X, Dahlin A, Evers R, Fischer V, Hillgren KM, Hoffmaster KA, Ishikawa T, Keppler D, Kim RB, Lee CA, Niemi M, Polli JW, Sugiyama Y, Swaan PW, Ware JA, Wright SH, Yee SW, Zamek-Gliszczynski MJ & Zhang L (2010) Membrane transporters in drug development. Nat Rev Drug Discov 9(3): 215–236.

Isin EM, Elmore CS, Nilsson GN, Thompson RA & Weidolf L (2012) Use of radiolabeled compounds in drug metabolism and pharmacokinetic studies. Chem Res Toxicol 25(3): 532–542.

Isoherranen N, Hachad H, Yeung CK & Levy RH (2009) Qualitative analysis of the role of metabolites in inhibitory drug-drug interactions: literature evaluation based on the metabolism and transport drug interaction database. Chem Res Toxicol 22(2): 294–298.

Jamei M, Turner D, Yang J, Neuhoff S, Polak S, Rostami-Hodjegan A & Tucker G (2009) Population-based mechanistic prediction of oral drug absorption. AAPS J 11(2): 225–237.

Jigorel E & Houston JB (2012) Utility of drug depletion-time profiles in isolated hepatocytes for accessing hepatic uptake clearance: identifying rate-limiting steps and role of passive processes. Drug Metab Dispos 40(8): 1596–1602.

Johansson I & Ingelman-Sundberg M (2011) Genetic polymorphism and toxicology–with emphasis on cytochrome p450. Toxicol Sci 120(1): 1–13.

Johnson TW, Dress KR & Edwards M (2009) Using the Golden Triangle to optimize clearance and oral absorption. Bioorg Med Chem Lett 19(19): 5560–5564.

Jones HM, Chen Y, Gibson C, Heimbach T, Parrott N, Peters SA, Snoeys J, Upreti VV, Zheng M & Hall SD (2015) Physiologically based pharmacokinetic modeling in drug discovery and development: a pharmaceutical industry perspective. Clin Pharmacol Ther 97(3): 247–262.

Jones HM, Dickins M, Youdim K, Gosset JR, Attkins NJ, Hay TL, Gurrell IK, Logan YR, Bungay PJ, Jones BC & Gardner IB (2012) Application of PBPK modelling in drug discovery and development at Pfizer. Xenobiotica 42(1): 94–106.

Jones HM, Gardner IB & Watson KJ (2009) Modelling and PBPK simulation in drug discovery. AAPS J 11(1): 155–166.

Joo J, Lee B, Lee T & Liu KH (2014) Screening of six UGT enzyme activities in human liver microsomes using liquid chromatography/triple quadrupole mass spectrometry. Rapid Commun Mass Spectrom 28(22): 2405–2414.

Kakkar AK & Dahiya N (2014) The evolving drug development landscape: from blockbusters to niche busters in the orphan drug space. Drug Development Research 75: 231–234.

Kalgutkar AS (2011) Handling reactive metabolite positives in drug discovery: What has retrospective structure-toxicity analyses taught us? Chem Biol Interact 192(1–2): 46–55.

Kalgutkar AS & Dalvie D (2015) Predicting toxicities of reactive metabolite-positive drug candidates. Annu Rev Pharmacol Toxicol 55: 35–54.

Kalgutkar AS & Didiuk MT (2009) Structural alerts, reactive metabolites, and protein covalent binding: how reliable are these attributes as predictors of drug toxicity? Chem Biodivers 6(11): 2115–2137.

Karlsson FH, Bouchene S, Hilgendorf C, Dolgos H & Peters SA (2013) Utility of in vitro systems and preclinical data for the prediction of human intestinal first-pass metabolism during drug discovery and preclinical development. Drug Metab Dispos 41(12): 2033–2046.

Katoh M & Yokoi T (2007) Application of chimeric mice with humanized liver for predictive ADME. Drug Metab Rev 39(1): 145–157.

Kennedy T (1997) Managing the drug discovery/development interface. Drug Discov Today 2(10): 436–444.

Keseru GM & Makara GM (2009) The influence of lead discovery strategies on the properties of drug candidates. Nat Rev Drug Discov 8(3): 203–212.

Khanna I (2012) Drug discovery in pharmaceutical industry: productivity challenges and trends. Drug Discov Today 17(19–20): 1088–1102.

Khetani SR & Bhatia SN (2008) Microscale culture of human liver cells for drug development. Nat Biotechnol 26(1): 120–126.

Kilford PJ, Gertz M, Houston JB & Galetin A (2008) Hepatocellular binding of drugs: correction for unbound fraction in hepatocyte incubations using microsomal binding or drug lipophilicity data. Drug Metab Dispos 36(7): 1194–1197.

Kinch MS, Haynesworth A, Kinch SL & Hoyer D (2014) An overview of FDA-approved new molecular entities: 1827–2013. Drug Discov Today 19(8): 1033–1039.

Kirchmair J, Goller AH, Lang D, Kunze J, Testa B, Wilson ID, Glen RC & Schneider G (2015) Predicting drug metabolism: experiment and/or computation? Nat Rev Drug Discov 14(6): 387–404.

Knights KM, Rowland A & Miners JO (2013) Renal drug metabolism in humans: the potential for drug-endobiotic interactions involving cytochrome P450 (CYP) and UDP-glucuronosyltransferase (UGT). Br J Clin Pharmacol 76(4): 587–602.

Kola I & Landis J (2004) Can the pharmaceutical industry reduce attrition rates? Nat Rev Drug Discov 3(8): 711–715.

Konig J, Muller F & Fromm MF (2013) Transporters and drug-drug interactions: important determinants of drug disposition and effects. Pharmacol Rev 65(3): 944–966.

Korjamo T, Monkkonen J, Uusitalo J, Turpeinen M, Pelkonen O & Honkakoski P (2006) Metabolic and efflux properties of Caco-2 cells stably transfected with nuclear receptors. Pharm Res 23(9): 1991–2001.

Koskimies P, Turunen J, Lammintausta R & Scheinin M (2013) Single-dose and steady-state pharmacokinetics of ospemifene, a selective estrogen receptor modulator, in postmenopausal women. Int J Clin Pharmacol Ther 51(11): 861–867.

Kosugi Y, Hirabayashi H, Igari T, Fujioka Y, Hara Y, Okuda T & Moriwaki T (2012) Evaluation of cytochrome P450-mediated drug-drug interactions based on the strategies recommended by regulatory authorities. Xenobiotica 42(2): 127–138.

Kramer JA, Sagartz JE & Morris DL (2007) The application of discovery toxicology and pathology towards the design of safer pharmaceutical lead candidates. Nat Rev Drug Discov 6(8): 636–649.

Kubinyi H (2003) Drug research: myths, hype and reality. Nat Rev Drug Discov 2(8): 665–668.

Kublbeck J, Laitinen T, Jyrkkarinne J, Rousu T, Tolonen A, Abel T, Kortelainen T, Uusitalo J, Korjamo T, Honkakoski P & Molnar F (2011) Use of comprehensive screening methods to detect selective human CAR activators. Biochem Pharmacol 82(12): 1994–2007.

Lagas JS, Sparidans RW, Wagenaar E, Beijnen JH & Schinkel AH (2010) Hepatic clearance of reactive glucuronide metabolites of diclofenac in the mouse is dependent on multiple ATP-binding cassette efflux transporters. Mol Pharmacol 77(4): 687–694.

Lammert C, Einarsson S, Saha C, Niklasson A, Bjornsson E & Chalasani N (2008) Relationship between daily dose of oral medications and idiosyncratic drug-induced liver injury: search for signals. Hepatology 47(6): 2003–2009.

Lappin G, Kuhnz W, Jochemsen R, Kneer J, Chaudhary A, Oosterhuis B, Drijfhout WJ, Rowland M & Garner RC (2006) Use of microdosing to predict pharmacokinetics at the therapeutic dose: experience with 5 drugs. Clin Pharmacol Ther 80(3): 203–215.

Lappin G, Shishikura Y, Jochemsen R, Weaver RJ, Gesson C, Brian Houston J, Oosterhuis B, Bjerrum OJ, Grynkiewicz G, Alder J, Rowland M & Garner C (2011) Comparative pharmacokinetics between a microdose and therapeutic dose for clarithromycin, sumatriptan, propafenone, paracetamol (acetaminophen), and phenobarbital in human volunteers. Eur J Pharm Sci 43(3): 141–150.

Lassila T, Mattila S, Turpeinen M & Tolonen A (2015) Glutathione trapping of reactive drug metabolites produced by biomimetic metalloporphyrin catalysts. Rapid Commun Mass Spectrom 29: 521–532.

Le Vee M, Jigorel E, Glaise D, Gripon P, Guguen-Guillouzo C & Fardel O (2006) Functional expression of sinusoidal and canalicular hepatic drug transporters in the differentiated human hepatoma HepaRG cell line. Eur J Pharm Sci 28(1–2): 109–117.

Leclercq L, Cuyckens F, Mannens GS, de Vries R, Timmerman P & Evans DC (2009) Which human metabolites have we MIST? Retrospective analysis, practical aspects, and perspectives for metabolite identification and quantification in pharmaceutical development. Chem Res Toxicol 22(2): 280–293.

Leeson PD & Springthorpe B (2007) The influence of drug-like concepts on decision-making in medicinal chemistry. Nat Rev Drug Discov 6(11): 881–890.

Lenz EM, Martin S, Schmidt R, Morin PE, Smith R, Weston DJ & Bayrakdarian M (2014) Reactive metabolite trapping screens and potential pitfalls: bioactivation of a homomorpholine and formation of an unstable thiazolidine adduct. Chem Res Toxicol 27(6): 968–980.

Lerner CA, Sundar IK, Watson RM, Elder A, Jones R, Done D, Kurtzman R, Ossip DJ, Robinson R, McIntosh S & Rahman I (2015) Environmental health hazards of e-cigarettes and their components: Oxidants and copper in e-cigarette aerosols. Environ Pollut 198C: 100–107.

Lexchin J (2014) How safe are new drugs? Market withdrawal of drugs approved in Canada between 1990 and 2009. Open Med 8(1): e14–9.

Li AC, Ding J, Jiang X & Denissen J (2009) Two-injection workflow for a liquid chromatography/LTQ-Orbitrap system to complete in vivo biotransformation characterization: demonstration with buspirone metabolite identification. Rapid Commun Mass Spectrom 23(18): 3003–3012.

Li AP (2007) Human hepatocytes: isolation, cryopreservation and applications in drug development. Chem Biol Interact 168(1): 16–29.

Li AP (2009) Overview: Evaluation of metabolism-based drug toxicity in drug development. Chem Biol Interact 179(1): 1–3.

Li Y, Zhou J, Ramsden D, Taub ME, O'Brien D, Xu J, Busacca CA, Gonnella N & Tweedie DJ (2014) Enzyme-transporter interplay in the formation and clearance of abundant metabolites of faldaprevir found in excreta but not in circulation. Drug Metab Dispos 42(3): 384–393.

Liberman RG, Tannenbaum SR, Hughey BJ, Shefer RE, Klinkowstein RE, Prakash C, Harriman SP & Skipper PL (2004) An interface for direct analysis of (14)c in nonvolatile samples by accelerator mass spectrometry. Anal Chem 76(2): 328–334.

Liebler DC (2008) Protein damage by reactive electrophiles: targets and consequences. Chem Res Toxicol 21(1): 117–128.

Lim HK, Chen J, Cook K, Sensenhauser C, Silva J & Evans DC (2008) A generic method to detect electrophilic intermediates using isotopic pattern triggered data-dependent high-resolution accurate mass spectrometry. Rapid Commun Mass Spectrom 22(8): 1295–1311.

Lim HK, Chen J, Sensenhauser C, Cook K & Subrahmanyam V (2007) Metabolite identification by data-dependent accurate mass spectrometric analysis at resolving power of 60,000 in external calibration mode using an LTQ/Orbitrap. Rapid Commun Mass Spectrom 21(12): 1821–1832.

Lin JH, Chiba M & Baillie TA (1999) Is the role of the small intestine in first-pass metabolism overemphasized? Pharmacol Rev 51(2): 135–158.

Lipinski CA, Lombardo F, Dominy BW & Feeney PJ (1997) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv Drug Deliv Rev 23: 3–25.

Liu DQ, Wu L, Sun M & MacGregor PA (2007) On-line H/D exchange LC-MS strategy for structural elucidation of pharmaceutical impurities. J Pharm Biomed Anal 44(2): 320–329.

Liu LY, Han YL, Zhu JH, Yu Q, Yang QJ, Lu J & Guo C (2015) A sensitive and high-throughput LC-MS/MS method for inhibition assay of seven major cytochrome P450s in human liver microsomes using an in vitro cocktail of probe substrates. Biomed Chromatogr 29(3): 437–444.

Loi CM, Smith DA & Dalvie D (2013) Which metabolites circulate? Drug Metab Dispos 41(5): 933–951.

Luffer-Atlas D (2008) Unique/major human metabolites: why, how, and when to test for safety in animals. Drug Metab Rev 40(3): 447–463.

Lutz JD & Isoherranen N (2012) Prediction of relative in vivo metabolite exposure from in vitro data using two model drugs: dextromethorphan and omeprazole. Drug Metab Dispos 40(1): 159–168.

Ma L, Wen B, Ruan Q & Zhu M (2008) Rapid screening of glutathione-trapped reactive metabolites by linear ion trap mass spectrometry with isotope pattern-dependent scanning and postacquisition data mining. Chem Res Toxicol 21(7): 1477–1483.

Ma S & Chowdhury SK (2011) Analytical strategies for assessment of human metabolites in preclinical safety testing. Anal Chem 83(13): 5028–5036.

Ma S & Chowdhury SK (2014) A tiered approach to address regulatory drug metabolite-related issues in drug development. Bioanalysis 6(5): 587–590.

Ma S, Li Z, Lee KJ & Chowdhury SK (2010) Determination of exposure multiples of human metabolites for MIST assessment in preclinical safety species without using reference standards or radiolabeled compounds. Chem Res Toxicol 23(12): 1871–1873.

Ma S & Zhu M (2009) Recent advances in applications of liquid chromatography-tandem mass spectrometry to the analysis of reactive drug metabolites. Chem Biol Interact 179(1): 25–37.

Ma X & Chan EC (2010) Fluorescence-based liver microsomal assay for screening of pharmaceutical reactive metabolites using a glutathione conjugated 96-well plate. Bioconjug Chem 21(1): 46–55.

Maeda K & Sugiyama Y (2011) Novel strategies for microdose studies using non-radiolabeled compounds. Adv Drug Deliv Rev 63(7): 532–538.

Manevski N, Swart P, Balavenkatraman KK, Bertschi B, Camenisch G, Kretz O, Schiller H, Walles M, Ling B, Wettstein R, Schaefer DJ, Itin P, Ashton-Chess J, Pognan F, Wolf A & Litherland K (2015) Phase II metabolism in human skin: skin explants show full coverage for glucuronidation, sulfation, N-acetylation, catechol methylation, and glutathione conjugation. Drug Metab Dispos 43(1): 126–139.

Mao J, Mohutsky MA, Harrelson JP, Wrighton SA & Hall SD (2011) Prediction of CYP3A-mediated drug-drug interactions using human hepatocytes suspended in human plasma. Drug Metab Dispos 39(4): 591–602.

Mao J, Mohutsky MA, Harrelson JP, Wrighton SA & Hall SD (2012) Predictions of cytochrome P450-mediated drug-drug interactions using cryopreserved human hepatocytes: comparison of plasma and protein-free media incubation conditions. Drug Metab Dispos 40(4): 706–716.

Masubuchi N, Makino C & Murayama N (2007) Prediction of in vivo potential for metabolic activation of drugs into chemically reactive intermediate: correlation of in vitro and in vivo generation of reactive intermediates and in vitro glutathione conjugate formation in rats and humans. Chem Res Toxicol 20(3): 455–464.

Mazzarino M, Biava M, de la Torre X, Fiacco I & Botre F (2013) Characterization of the biotransformation pathways of clomiphene, tamoxifen and toremifene as assessed by LC-MS/(MS) following in vitro and excretion studies. Anal Bioanal Chem 405(16): 5467–5487.

McNaughton R, Huet G & Shakir S (2014) An investigation into drug products withdrawn from the EU market between 2002 and 2011 for safety reasons and the evidence used to support the decision-making. BMJ Open 4(1): e004221–2013–004221.

Meanwell NA (2011) Improving drug candidates by design: a focus on physicochemical properties as a means of improving compound disposition and safety. Chem Res Toxicol 24(9): 1420–1456.

Meneses-Lorente G, Sakatis MZ, Schulz-Utermoehl T, De Nardi C & Watt AP (2006) A quantitative high-throughput trapping assay as a measurement of potential for bioactivation. Anal Biochem 351(2): 266–272.

Mitchell MD, Elrick MM, Walgren JL, Mueller RA, Morris DL & Thompson DC (2008) Peptide-based in vitro assay for the detection of reactive metabolites. Chem Res Toxicol 21(4): 859–868.

Miura M, Hori W, Kasahara Y & Nakagawa I (2010) Quantitative assessment of the metabolic activation of alicyclic amines via aldehyde. J Pharmacol Toxicol Methods 61(1): 44–51.

Morgan P, Van Der Graaf PH, Arrowsmith J, Feltner DE, Drummond KS, Wegner CD & Street SD (2012) Can the flow of medicines be improved? Fundamental pharmacokinetic and pharmacological principles toward improving Phase II survival. Drug Discov Today 17(9–10): 419–424.

Morphy R (2006) The influence of target family and functional activity on the physicochemical properties of pre-clinical compounds. J Med Chem 49(10): 2969–2978.

Mortishire-Smith RJ, O'Connor D, Castro-Perez JM & Kirby J (2005) Accelerated throughput metabolic route screening in early drug discovery using high-resolution liquid chromatography/quadrupole time-of-flight mass spectrometry and automated data analysis. Rapid Commun Mass Spectrom 19(18): 2659–2670.

Muchmore SW, Edmunds JJ, Stewart KD & Hajduk PJ (2010) Cheminformatic tools for medicinal chemists. J Med Chem 53(13): 4830–4841.

Mullard A (2014a) 2013 FDA drug approvals. Nat Rev Drug Discov 13(2): 85–89.

Mullard A (2014b) 2013 in Reflection. Nat Rev Drug Discov 13(1): 5–7.

Mullard A (2015) 2014 FDA drug approvals. Nat Rev Drug Discov 14(2): 77–81.

Muller PY (2011) Comparative requirements for exploratory clinical trials – eIND, eCTA and microdosing. Adv Drug Deliv Rev 63(7): 511–517.

Munos B (2009) Lessons from 60 years of pharmaceutical innovation. Nat Rev Drug Discov 8(12): 959–968.

Mutlib A, Espina R, Vishwanathan K, Babalola K, Chen Z, Dehnhardt C, Venkatesan A, Mansour T, Chaudhary I, Talaat R & Scatina J (2011) Application of quantitative NMR in pharmacological evaluation of biologically generated metabolites: implications in drug discovery. Drug Metab Dispos 39(1): 106–116.

Naito S, Furuta S, Yoshida T, Kitada M, Fueki O, Unno T, Ohno Y, Onodera H, Kawamura N, Kurokawa M, Sagami F, Shinoda K, Nakazawa T & Yamazaki T (2007) Current opinion: safety evaluation of drug metabolites in development of pharmaceuticals. J Toxicol Sci 32(4): 329–341.

Nakayama S, Atsumi R, Takakusa H, Kobayashi Y, Kurihara A, Nagai Y, Nakai D & Okazaki O (2009) A zone classification system for risk assessment of idiosyncratic drug toxicity using daily dose and covalent binding. Drug Metab Dispos 37(9): 1970–1977.

Nakayama S, Takakusa H, Watanabe A, Miyaji Y, Suzuki W, Sugiyama D, Shiosakai K, Honda K, Okudaira N, Izumi T & Okazaki O (2011) Combination of GSH trapping and time-dependent inhibition assays as a predictive method of drugs generating highly reactive metabolites. Drug Metab Dispos 39(7): 1247–1254.

Nappi RE, Panay N, Bruyniks N, Castelo-Branco C, De Villiers TJ & Simon JA (2015) The clinical relevance of the effect of ospemifene on symptoms of vulvar and vaginal atrophy. Climacteric 18(2): 233–240.

Nassar AE, Kamel AM & Clarimont C (2004) Improving the decision-making process in the structural modification of drug candidates: enhancing metabolic stability. Drug Discov Today 9(23): 1020–1028.

Nedderman AN (2009) Metabolites in safety testing: metabolite identification strategies in discovery and development. Biopharm Drug Dispos 30(4): 153–162.

Nedderman AN, Dear GJ, North S, Obach RS & Higton D (2011) From definition to implementation: a cross-industry perspective of past, current and future MIST strategies. Xenobiotica 41(8): 605–622.

Nigam SK (2015) What do drug transporters really do? Nat Rev Drug Discov 14(1): 29–44.

Obach RS (2013) Pharmacologically active drug metabolites: impact on drug discovery and pharmacotherapy. Pharmacol Rev 65(2): 578–640.

Obach RS, Kalgutkar AS, Soglia JR & Zhao SX (2008b) Can in vitro metabolism-dependent covalent binding data in liver microsomes distinguish hepatotoxic from nonhepatotoxic drugs? An analysis of 18 drugs with consideration of intrinsic clearance and daily dose. Chem Res Toxicol 21(9): 1814–1822.

Obach RS, Lombardo F & Waters NJ (2008a) Trend analysis of a database of intravenous pharmacokinetic parameters in humans for 670 drug compounds. Drug Metab Dispos 36(7): 1385–1405.

Obach RS, Nedderman AN & Smith DA (2012) Radiolabelled mass-balance excretion and metabolism studies in laboratory animals: are they still necessary? Xenobiotica 42(1): 46–56.

Obach RS, Nedderman AN & Smith DA (2013) A response to "radiolabelled mass-balance excretion and metabolism studies in laboratory animals: a commentary on why they are still necessary". Xenobiotica 43(2): 226–227.

Oda S, Fukami T, Yokoi T & Nakajima M (2015) A comprehensive review of UDP-glucuronosyltransferase and esterases for drug development. Drug Metab Pharmacokinet 30(1): 30–51.

Olson H, Betton G, Robinson D, Thomas K, Monro A, Kolaja G, Lilly P, Sanders J, Sipes G, Bracken W, Dorato M, Van Deun K, Smith P, Berger B & Heller A (2000) Concordance of the toxicity of pharmaceuticals in humans and in animals. Regul Toxicol Pharmacol 32(1): 56–67.

Orr ST, Ripp SL, Ballard TE, Henderson JL, Scott DO, Obach RS, Sun H & Kalgutkar AS (2012) Mechanism-based inactivation (MBI) of cytochrome P450 enzymes: structure-activity relationships and discovery strategies to mitigate drug-drug interaction risks. J Med Chem 55(11): 4896–4933.

Owens PK, Raddad E, Miller JW, Stille JR, Olovich KG, Smith NV, Jones RS & Scherer JC (2015) A decade of innovation in pharmaceutical R&D: the Chorus model. Nat Rev Drug Discov 14(1): 17–28.

Paine MF, Hart HL, Ludington SS, Haining RL, Rettie AE & Zeldin DC (2006) The human intestinal cytochrome P450 "pie". Drug Metab Dispos 34(5): 880–886.

Pang KS (2009) Safety testing of metabolites: Expectations and outcomes. Chem Biol Interact 179(1): 45–59.

Park BK, Boobis A, Clarke S, Goldring CE, Jones D, Kenna JG, Lambert C, Laverty HG, Naisbitt DJ, Nelson S, Nicoll-Griffith DA, Obach RS, Routledge P, Smith DA, Tweedie DJ, Vermeulen N, Williams DP, Wilson ID & Baillie TA (2011a) Managing the challenge of chemically reactive metabolites in drug development. Nat Rev Drug Discov 10(4): 292–306.

Park BK, Kitteringham NR, Powell H & Pirmohamed M (2000) Advances in molecular toxicology-towards understanding idiosyncratic drug toxicity. Toxicology 153(1–3): 39–60.

Park BK, Laverty H, Srivastava A, Antoine DJ, Naisbitt D & Williams DP (2011b) Drug bioactivation and protein adduct formation in the pathogenesis of drug-induced toxicity. Chem Biol Interact 192(1–2): 30–36.

Park BK, Pirmohamed M & Kitteringham NR (1998) Role of drug disposition in drug hypersensitivity: a chemical, molecular, and clinical perspective. Chem Res Toxicol 11(9): 969–988.

Parkinson A, Kazmi F, Buckley DB, Yerino P, Ogilvie BW & Paris BL (2010) System-dependent outcomes during the evaluation of drug candidates as inhibitors of cytochrome P450 (CYP) and uridine diphosphate glucuronosyltransferase (UGT) enzymes: human hepatocytes versus liver microsomes versus recombinant enzymes. Drug Metab Pharmacokinet 25(1): 16–27.

Paul SM, Mytelka DS, Dunwiddie CT, Persinger CC, Munos BH, Lindborg SR & Schacht AL (2010) How to improve R&D productivity: the pharmaceutical industry's grand challenge. Nat Rev Drug Discov 9(3): 203–214.

Pelkonen O, Kaltiala EH, Larmi TK & Karki NT (1974) Cytochrome P-450-linked monooxygenase system and drug-induced spectral interactions in human liver microsomes. Chem Biol Interact 9(3): 205–216.

Pelkonen O & Raunio H (2005) In vitro screening of drug metabolism during drug development: can we trust the predictions? Expert Opin Drug Metab Toxicol 1(1): 49–59.

Pelkonen O, Tolonen A, Korjamo T, Turpeinen M & Raunio H (2009a) From known knowns to known unknowns: predicting in vivo drug metabolites. Bioanalysis 1(2): 393–414.

Pelkonen O, Tolonen A, Rousu T, Tursas L, Turpeinen M, Hokkanen J, Uusitalo J, Bouvier d'Yvoire M & Coecke S (2009b) Comparison of metabolic stability and metabolite identification of 55 ECVAM/ICCVAM validation compounds between human and rat liver homogenates and microsomes – a preliminary analysis. ALTEX 26(3): 214–222.

Pelkonen O, Tolonen A, Turpeinen M & Uusitalo J (2008b) In vitro Metabolism in Preclinical Drug Development. In: Gad SC (ed) Preclinical Development Handbook: ADME and Biopharmaceutical Properties. Hoboken, New Jersey, John Wiley & Sons, Inc.: 743–774.

Pelkonen O, Turpeinen M, Hakkola J, Honkakoski P, Hukkanen J & Raunio H (2008a) Inhibition and induction of human cytochrome P450 enzymes: current status. Arch Toxicol 82(10): 667–715.

Pelkonen O, Turpeinen M & Raunio H (2011) In vivo-in vitro-in silico pharmacokinetic modelling in drug development: current status and future directions. Clin Pharmacokinet 50(8): 483–491.

Pelkonen O, Turpeinen M, Uusitalo J, Rautio A & Raunio H (2005) Prediction of drug metabolism and interactions on the basis of in vitro investigations. Basic Clin Pharmacol Toxicol 96(3): 167–175.

Penner N, Klunk LJ & Prakash C (2009) Human radiolabeled mass balance studies: objectives, utilities and limitations. Biopharm Drug Dispos 30(4): 185–203.

Penner N, Xu L & Prakash C (2012) Radiolabeled absorption, distribution, metabolism, and excretion studies in drug development: why, when, and how? Chem Res Toxicol 25(3): 513–531.

Perola E (2010) An analysis of the binding efficiencies of drugs and their leads in successful drug discovery programs. J Med Chem 53(7): 2986–2997.

Pinkerton JV & Thomas S (2014) Use of SERMs for treatment in postmenopausal women. J Steroid Biochem Mol Biol 142: 142–154.

Portman D, Palacios S, Nappi RE & Mueck AO (2014) Ospemifene, a non-oestrogen selective oestrogen receptor modulator for the treatment of vaginal dryness associated with postmenopausal vulvar and vaginal atrophy: a randomised, placebo-controlled, phase III trial. Maturitas 78(2): 91–98.

Poulin P, Kenny JR, Hop CE & Haddad S (2012) In vitro-in vivo extrapolation of clearance: modeling hepatic metabolic clearance of highly bound drugs and comparative assessment with existing calculation methods. J Pharm Sci 101(2): 838–851.

Powley MW, Frederick CB, Sistare FD & DeGeorge JJ (2009) Safety assessment of drug metabolites: implications of regulatory guidance and potential application of genetically engineered mouse models that express human P450s. Chem Res Toxicol 22(2): 257–262.

Prakash C, Li Z, Orlandi C & Klunk L (2012) Assessment of exposure of metabolites in preclinical species and humans at steady state from the single-dose radiolabeled absorption, distribution, metabolism, and excretion studies: a case study. Drug Metab Dispos 40(7): 1308–1320.

Prakash C, Shaffer CL & Nedderman A (2007) Analytical strategies for identifying drug metabolites. Mass Spectrom Rev 26(3): 340–369.

Prentis RA, Lis Y & Walker SR (1988) Pharmaceutical innovation by the seven UK-owned pharmaceutical companies (1964–1985). Br J Clin Pharmacol 25(3): 387–396.

Pritchard JF, Jurima-Romet M, Reimer ML, Mortimer E, Rolfe B & Cayen MN (2003) Making better drugs: Decision gates in non-clinical drug development. Nat Rev Drug Discov 2(7): 542–553.

Prueksaritanont T, Chu X, Gibson C, Cui D, Yee KL, Ballard J, Cabalu T & Hochman J (2013) Drug-drug interaction studies: regulatory guidance and an industry perspective. AAPS J 15(3): 629–645.

Prueksaritanont T, Lin JH & Baillie TA (2006) Complicating factors in safety testing of drug metabolites: kinetic differences between generated and preformed metabolites. Toxicol Appl Pharmacol 217(2): 143–152.

Qu Q, Zheng H, Dahllund J, Laine A, Cockcroft N, Peng Z, Koskinen M, Hemminki K, Kangas L, Vaananen K & Harkonen P (2000) Selective estrogenic effects of a novel triphenylethylene compound, FC1271a, on bone, cholesterol level, and reproductive tissues in intact and ovariectomized rats. Endocrinology 141(2): 809–820.

Rathahao-Paris E, Paris A, Bursztyka J, Jaeg JP, Cravedi JP & Debrauwer L (2014) Identification of xenobiotic metabolites from biological fluids using flow injection analysis high-resolution mass spectrometry and post-acquisition data filtering. Rapid Commun Mass Spectrom 28(24): 2713–2722.

Ravindran S, Jadhav A, Surve P, Lonsane G, Honrao P & Nanda B (2014) Technologies and strategies to characterize and quantitate metabolites in drug discovery and development. Biomed Chromatogr 28(11): 1547–1553.

Reese M, Sakatis M, Ambroso J, Harrell A, Yang E, Chen L, Taylor M, Baines I, Zhu L, Ayrton A & Clarke S (2011) An integrated reactive metabolite evaluation approach to assess and reduce safety risk during drug discovery and development. Chem Biol Interact 192(1–2): 60–64.

Rendic SP & Guengerich FP (2015) Survey of Human Oxidoreductases and Cytochrome P450 Enzymes Involved in the Metabolism of Xenobiotic and Natural Chemicals. Chem Res Toxicol 28: 38–42.

Riccardi K, Cawley S, Yates PD, Chang C, Funk C, Niosi M, Lin J & Di L (2015) Plasma Protein Binding of Challenging Compounds. J Pharm Sci : 10.1002/jps.24506 [doi].

Roffey SJ, Obach RS, Gedge JI & Smith DA (2007) What is the objective of the mass balance study? A retrospective analysis of data in animal and human excretion studies employing radiolabeled drugs. Drug Metab Rev 39(1): 17–43.

Rostami-Hodjegan A (2012) Physiologically based pharmacokinetics joined with in vitro-in vivo extrapolation of ADME: a marriage under the arch of systems pharmacology. Clin Pharmacol Ther 92(1): 50–61.

Rostami-Hodjegan A, Tamai I & Pang KS (2012) Physiologically based pharmacokinetic (PBPK) modeling: it is here to stay! Biopharm Drug Dispos 33(2): 47–50.

Rostami-Hodjegan A & Tucker GT (2007) Simulation and prediction of in vivo drug metabolism in human populations from in vitro data. Nat Rev Drug Discov 6(2): 140–148.

Rousu T, Pelkonen O & Tolonen A (2009) Rapid detection and characterization of reactive drug metabolites in vitro using several isotope-labeled trapping agents and ultra-performance liquid chromatography/time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 23(6): 843–855.

Rousu T & Tolonen A (2011) Characterization of cyanide-trapped methylated metabonates formed during reactive drug metabolite screening in vitro. Rapid Commun Mass Spectrom 25(10): 1382–1390.

Ruan Q, Peterman S, Szewc MA, Ma L, Cui D, Humphreys WG & Zhu M (2008) An integrated method for metabolite detection and identification using a linear ion trap/Orbitrap mass spectrometer and multiple data processing techniques: application to indinavir metabolite detection. J Mass Spectrom 43(2): 251–261.

Sakatis MZ, Reese MJ, Harrell AW, Taylor MA, Baines IA, Chen L, Bloomer JC, Yang EY, Ellens HM, Ambroso JL, Lovatt CA, Ayrton AD & Clarke SE (2012) Preclinical strategy to reduce clinical hepatotoxicity using in vitro bioactivation data for >200 compounds. Chem Res Toxicol 25(10): 2067–2082.

Salehpour M, Forsgard N & Possnert G (2009) FemtoMolar measurements using accelerator mass spectrometry. Rapid Commun Mass Spectrom 23(5): 557–563.

Samuel K, Yin W, Stearns RA, Tang YS, Chaudhary AG, Jewell JP, Lanza T,Jr, Lin LS, Hagmann WK, Evans DC & Kumar S (2003) Addressing the metabolic activation potential of new leads in drug discovery: a case study using ion trap mass spectrometry and tritium labeling techniques. J Mass Spectrom 38(2): 211–221.

Sanoh S & Ohta S (2014) Chimeric mice transplanted with human hepatocytes as a model for prediction of human drug metabolism and pharmacokinetics. Biopharm Drug Dispos 35(2): 71–86.

Sanoh S, Tayama Y, Sugihara K, Kitamura S & Ohta S (2015) Significance of aldehyde oxidase during drug development: Effects on drug metabolism, pharmacokinetics, toxicity, and efficacy. Drug Metab Pharmacokinet 30(1): 52–63.

Sawamura R, Okudaira N, Watanabe K, Murai T, Kobayashi Y, Tachibana M, Ohnuki T, Masuda K, Honma H, Kurihara A & Okazaki O (2010) Predictability of idiosyncratic drug toxicity risk for carboxylic acid-containing drugs based on the chemical stability of acyl glucuronide. Drug Metab Dispos 38(10): 1857–1864.

Scannell JW, Blanckley A, Boldon H & Warrington B (2012) Diagnosing the decline in pharmaceutical R&D efficiency. Nat Rev Drug Discov 11(3): 191–200.

Schuetz JD, Swaan PW & Tweedie DJ (2014) The role of transporters in toxicity and disease. Drug Metab Dispos 42(4): 541–545.

Shaffer CL, Scialis RJ, Rong H & Obach RS (2012) Using Simcyp to project human oral pharmacokinetic variability in early drug research to mitigate mechanism-based adverse events. Biopharm Drug Dispos 33(2): 72–84.

Shah F & Greene N (2014) Analysis of Pfizer compounds in EPA's ToxCast chemicals-assay space. Chem Res Toxicol 27(1): 86–98.

Shardlow CE, Generaux GT, MacLauchlin CC, Pons N, Skordos KW & Bloomer JC (2011) Utilizing drug-drug interaction prediction tools during drug development: enhanced decision making based on clinical risk. Drug Metab Dispos 39(11): 2076–2084.

Sharma R, Litchfield J, Atkinson K, Eng H, Amin NB, Denney WS, Pettersen JC, Goosen TC, Di L, Lee E, Pfefferkorn JA, Dalvie DK & Kalgutkar AS (2014) Metabolites in safety testing assessment in early clinical development: a case study with a glucokinase activator. Drug Metab Dispos 42(11): 1926–1939.

Sharma R, Litchfield J, Bergman A, Atkinson K, Kazierad D, Gustavson SM, Di L, Pfefferkorn JA & Kalgutkar AS (2015) Comparison of the circulating metabolite profile of PF-04991532, a hepatoselective glucokinase activator, across preclinical species and humans: potential implications in metabolites in safety testing assessment. Drug Metab Dispos 43(2): 190–198.

Shimizu A, Ohe T & Chiba M (2012) A novel method for the determination of the site of glucuronidation by ion mobility spectrometry-mass spectrometry. Drug Metab Dispos 40(8): 1456–1459.

Shinde P, Patil P, Mandhare M, Joshi Y, Patil L & Kadam V (2011) Microdosing: a new strategy for drug development process. Int J Pharm Sci &Res 2(7): 1836–1849.

Shu YZ, Johnson BM & Yang TJ (2008) Role of biotransformation studies in minimizing metabolism-related liabilities in drug discovery. AAPS J 10(1): 178–192.

Simon J, Portman D, Mabey RG,Jr & Ospemifene Study Group (2014) Long-term safety of ospemifene (52-week extension) in the treatment of vulvar and vaginal atrophy in hysterectomized postmenopausal women. Maturitas 77(3): 274–281.

Singh D, Kashyap A, Pandey RV & Saini KS (2011) Novel advances in cytochrome P450 research. Drug Discov Today 16(17–18): 793–799.

Sinha V, Zhao P, Huang SM & Zineh I (2014) Physiologically based pharmacokinetic modeling: from regulatory science to regulatory policy. Clin Pharmacol Ther 95(5): 478–480.

Sinha VK, Snoeys J, Osselaer NV, Peer AV, Mackie C & Heald D (2012) From preclinical to human–prediction of oral absorption and drug-drug interaction potential using physiologically based pharmacokinetic (PBPK) modeling approach in an industrial setting: a workflow by using case example. Biopharm Drug Dispos 33(2): 111–121.

Sipilä H, Kangas L, Vuorilehto L, Kalapudas A, Eloranta M, Södervall M, Toivola R & Anttila M (1990) Metabolism of toremifene in the rat. J Steroid Biochem 36(3): 211–215.

Smietana K, Ekstrom L, Jeffery B & Moller M (2015) Improving R&D productivity. Nat Rev Drug Discov 14(7): 455–456.
Smith BJ (2012) An industrial perspective on contemporary applications of PBPK models in drug discovery and development. Biopharm Drug Dispos 33(2): 53–54.
Smith DA (2009) Dogma driven science, the need to establish a common base line. Chem Biol Interact 179(1): 68–70.
Smith DA (2013) Evolution of ADME science: where else can modeling and simulation contribute? Mol Pharm 10(4): 1162–1170.
Smith DA & Dalvie D (2012) Why do metabolites circulate? Xenobiotica 42(1): 107–126.
Smith DA, Di L & Kerns EH (2010) The effect of plasma protein binding on in vivo efficacy: misconceptions in drug discovery. Nat Rev Drug Discov 9(12): 929–939.
Smith DA & Obach RS (2005) Seeing through the mist: abundance versus percentage. Commentary on metabolites in safety testing. Drug Metab Dispos 33(10): 1409–1417.
Smith DA & Obach RS (2006) Metabolites and safety: What are the concerns, and how should we address them? Chem Res Toxicol 19(12): 1570–1579.
Smith DA & Obach RS (2009) Metabolites in safety testing (MIST): considerations of mechanisms of toxicity with dose, abundance, and duration of treatment. Chem Res Toxicol 22(2): 267–279.
Smith DA, Obach RS, Williams DP & Park BK (2009) Clearing the MIST (metabolites in safety testing) of time: The impact of duration of administration on drug metabolite toxicity. Chem Biol Interact 179(1): 60–67.
Smith RW, Toutoungi DE, Reynolds JC, Bristow AW, Ray A, Sage A, Wilson ID, Weston DJ, Boyle B & Creaser CS (2013) Enhanced performance in the determination of ibuprofen 1-beta-O-acyl glucuronide in urine by combining high field asymmetric waveform ion mobility spectrometry with liquid chromatography-time-of-flight mass spectrometry. J Chromatogr A 1278: 76–81.
Soars MG, McGinnity DF, Grime K & Riley RJ (2007) The pivotal role of hepatocytes in drug discovery. Chem Biol Interact 168(1): 2–15.
Soglia JR, Contillo LG, Kalgutkar AS, Zhao S, Hop CE, Boyd JG & Cole MJ (2006) A semiquantitative method for the determination of reactive metabolite conjugate levels in vitro utilizing liquid chromatography-tandem mass spectrometry and novel quaternary ammonium glutathione analogues. Chem Res Toxicol 19(3): 480–490.
Sousa T, Paterson R, Moore V, Carlsson A, Abrahamsson B & Basit AW (2008) The gastrointestinal microbiota as a site for the biotransformation of drugs. Int J Pharm 363(1–2): 1–25.
Srivastava A, Lian LY, Maggs JL, Chaponda M, Pirmohamed M, Williams DP & Park BK (2010) Quantifying the metabolic activation of nevirapine in patients by integrated applications of NMR and mass spectrometries. Drug Metab Dispos 38(1): 122–132.
Stachulski AV, Baillie TA, Park BK, Obach RS, Dalvie DK, Williams DP, Srivastava A, Regan SL, Antoine DJ, Goldring CE, Chia AJ, Kitteringham NR, Randle LE, Callan H, Castrejon JL, Farrell J, Naisbitt DJ & Lennard MS (2013) The generation, detection, and effects of reactive drug metabolites. Med Res Rev 33(5): 985–1080.

Stepan AF, Walker DP, Bauman J, Price DA, Baillie TA, Kalgutkar AS & Aleo MD (2011) Structural alert/reactive metabolite concept as applied in medicinal chemistry to mitigate the risk of idiosyncratic drug toxicity: a perspective based on the critical examination of trends in the top 200 drugs marketed in the United States. Chem Res Toxicol 24(9): 1345–1410.

Stringer R, Nicklin PL & Houston JB (2008) Reliability of human cryopreserved hepatocytes and liver microsomes as in vitro systems to predict metabolic clearance. Xenobiotica 38(10): 1313–1329.

Sugiyama Y & Yamashita S (2011) Impact of microdosing clinical study – why necessary and how useful? Adv Drug Deliv Rev 63(7): 494–502.

Tachibana T, Kato M, Watanabe T, Mitsui T & Sugiyama Y (2009) Method for predicting the risk of drug-drug interactions involving inhibition of intestinal CYP3A4 and P-glycoprotein. Xenobiotica 39(6): 430–443.

Takakusa H, Masumoto H, Makino C, Okazaki O & Sudo K (2009) Quantitative assessment of reactive metabolite formation using 35S-labeled glutathione. Drug Metab Pharmacokinet 24(1): 100–107.

Takakusa H, Masumoto H, Yukinaga H, Makino C, Nakayama S, Okazaki O & Sudo K (2008) Covalent binding and tissue distribution/retention assessment of drugs associated with idiosyncratic drug toxicity. Drug Metab Dispos 36(9): 1770–1779.

Tang W & Lu AY (2010) Metabolic bioactivation and drug-related adverse effects: current status and future directions from a pharmaceutical research perspective. Drug Metab Rev 42(2): 225–249.

Testa B (2009) Drug metabolism for the perplexed medicinal chemist. Chem Biodivers 6(11): 2055–2070.

Testa B & Kramer SD (2009) The biochemistry of drug metabolism – an introduction: part 5. Metabolism and bioactivity. Chem Biodivers 6(5): 591–684.

Testa B, Pedretti A & Vistoli G (2012) Reactions and enzymes in the metabolism of drugs and other xenobiotics. Drug Discov Today 17(11–12): 549–560.

Thompson RA, Isin EM, Li Y, Weaver R, Weidolf L, Wilson I, Claesson A, Page K, Dolgos H & Kenna JG (2011) Risk assessment and mitigation strategies for reactive metabolites in drug discovery and development. Chem Biol Interact 192(1–2): 65–71.

Thompson RA, Isin EM, Li Y, Weidolf L, Page K, Wilson I, Swallow S, Middleton B, Stahl S, Foster AJ, Dolgos H, Weaver R & Kenna JG (2012) In vitro approach to assess the potential for risk of idiosyncratic adverse reactions caused by candidate drugs. Chem Res Toxicol 25(8): 1616–1632.

Tiller PR, Yu S, Bateman KP, Castro-Perez J, McIntosh IS, Kuo Y & Baillie TA (2008b) Fractional mass filtering as a means to assess circulating metabolites in early human clinical studies. Rapid Commun Mass Spectrom 22(22): 3510–3516.

Tiller PR, Yu S, Castro-Perez J, Fillgrove KL & Baillie TA (2008a) High-throughput, accurate mass liquid chromatography/tandem mass spectrometry on a quadrupole time-of-flight system as a 'first-line' approach for metabolite identification studies. Rapid Commun Mass Spectrom 22(7): 1053–1061.

Timmerman P, Anders Kall M, Gordon B, Laakso S, Freisleben A & Hucker R (2010) Best practices in a tiered approach to metabolite quantification: views and recommendations of the European Bioanalysis Forum. Bioanalysis 2(7): 1185–1194.

Tolonen A & Pelkonen O (2015) Analytical challenges for conducting rapid metabolism characterization for QIVIVE. Toxicology 332: 20–29.

Tolonen A, Petsalo A, Turpeinen M, Uusitalo J & Pelkonen O (2007) In vitro interaction cocktail assay for nine major cytochrome P450 enzymes with 13 probe reactions and a single LC/MSMS run: analytical validation and testing with monoclonal anti-CYP antibodies. J Mass Spectrom 42(7): 960–966.

Tolonen A, Turpeinen M & Pelkonen O (2009) Liquid chromatography–mass spectrometry in in vitro drug metabolite screening. Drug Discov Today 14(3–4): 120–133.

Tolonen A, Turpeinen M, Uusitalo J & Pelkonen O (2005) A simple method for differentiation of monoisotopic drug metabolites with hydrogen-deuterium exchange liquid chromatography/electrospray mass spectrometry. Eur J Pharm Sci 25(1): 155–162.

Tong W, Chowdhury SK, Su AD & Alton KB (2010) Quantitation of parent drug and its unstable metabolites by in situ coulometric oxidation and liquid chromatography-tandem mass spectrometry. Anal Chem 82(24): 10251–10257.

Tse MS & Kirkpatrick P (2013) News & Analysis: 2012 in reflection. Nat Rev Drug Discov 12(1): 8–10.

Turpeinen M, Ghiciuc C, Opritoui M, Tursas L, Pelkonen O & Pasanen M (2007) Predictive value of animal models for human cytochrome P450 (CYP)-mediated metabolism: a comparative study in vitro. Xenobiotica 37(12): 1367–1377.

Turpeinen M, Korhonen LE, Tolonen A, Uusitalo J, Juvonen R, Raunio H & Pelkonen O (2006) Cytochrome P450 (CYP) inhibition screening: comparison of three tests. Eur J Pharm Sci 29(2): 130–138.

Turpeinen M, Tolonen A, Chesne C, Guillouzo A, Uusitalo J & Pelkonen O (2009) Functional expression, inhibition and induction of CYP enzymes in HepaRG cells. Toxicol In Vitro 23(4): 748–753.

Turpeinen M, Uusitalo J, Jalonen J & Pelkonen O (2005) Multiple P450 substrates in a single run: rapid and comprehensive in vitro interaction assay. Eur J Pharm Sci 24(1): 123–132.

Uetrecht J (2003) Screening for the potential of a drug candidate to cause idiosyncratic drug reactions. Drug Discov Today 8(18): 832–837.

Uetrecht J (2006) Evaluation of which reactive metabolite, if any, is responsible for a specific idiosyncratic reaction. Drug Metab Rev 38(4): 745–753.

Uetrecht J (2007) Idiosyncratic drug reactions: current understanding. Annu Rev Pharmacol Toxicol 47: 513–539.

Uetrecht J (2008) Idiosyncratic drug reactions: past, present, and future. Chem Res Toxicol 21(1): 84–92.

Uetrecht J & Naisbitt DJ (2013) Idiosyncratic adverse drug reactions: current concepts. Pharmacol Rev 65(2): 779–808.

Unkila M, Kari S, Yatkin E & Lammintausta R (2013) Vaginal effects of ospemifene in the ovariectomized rat preclinical model of menopause. J Steroid Biochem Mol Biol 138: 107–115.

US Department of Health and Human Services, Food and Drug Administration, Center for Evaluation and Research (CDER) (2006) Guidance for Industry, Investigators, and Reviewers. Exploratory IND Studies. (January 2006).

US Department of Health and Human Services, Food and Drug Administration, Center for Evaluation and Research (CDER) (2008) Guidance for Industry, Safety Testing of Drug Metabolites. (February 2008).

US Department of Health and Human Services, Food and Drug Administration, Center for Evaluation and Research (CDER) (2012) Guidance for Industry, Drug Interaction Studies – Study Design, Data Analysis, Implications for Dosing, and Labeling Recommendations, Draft Guidance. February 2012.

US Department of Health and Human Services, Food and Drug Administration, Center for Evaluation and Research (CDER) (2014) Approved drugs 2013. http://www fda gov/downloads/Drugs/DevelopmentApprovalProcess/DrugInnovation/UCM381803 pdf Accessed 10 Feb 2015.

US Department of Health and Human Services, Food and Drug Administration, Center for Evaluation and Research (CDER) (2015) Novel new drugs 2014 summary. http://www fda gov/downloads/Drugs/DevelopmentApprovalProcess/DrugInnovation/UCM430299 pdf : Accessed 14 May 2015.

Usui T, Mise M, Hashizume T, Yabuki M & Komuro S (2009) Evaluation of the potential for drug-induced liver injury based on in vitro covalent binding to human liver proteins. Drug Metab Dispos 37(12): 2383–2392.

van de Waterbeemd H & Gifford E (2003) ADMET in silico modelling: towards prediction paradise? Nat Rev Drug Discov 2(3): 192–204.

VandenBrink BM & Isoherranen N (2010) The role of metabolites in predicting drug-drug interactions: focus on irreversible cytochrome P450 inhibition. Curr Opin Drug Discov Devel 13(1): 66–77.

Varma MV, Feng B, Obach RS, Troutman MD, Chupka J, Miller HR & El-Kattan A (2009) Physicochemical determinants of human renal clearance. J Med Chem 52(15): 4844–4852.

Varma MV, Obach RS, Rotter C, Miller HR, Chang G, Steyn SJ, El-Kattan A & Troutman MD (2010) Physicochemical space for optimum oral bioavailability: contribution of human intestinal absorption and first-pass elimination. J Med Chem 53(3): 1098–1108.

Varma MV, Pang KS, Isoherranen N & Zhao P (2015) Dealing with the complex drug-drug interactions: Towards mechanistic models. Biopharm Drug Dispos 36(2): 71–92.

Vieira ML, Zhao P, Berglund EG, Reynolds KS, Zhang L, Lesko LJ & Huang SM (2012) Predicting drug interaction potential with a physiologically based pharmacokinetic model: a case study of telithromycin, a time-dependent CYP3A inhibitor. Clin Pharmacol Ther 91(4): 700–708.

Vishwanathan K, Babalola K, Wang J, Espina R, Yu L, Adedoyin A, Talaat R, Mutlib A & Scatina J (2009) Obtaining exposures of metabolites in preclinical species through plasma pooling and quantitative NMR: addressing metabolites in safety testing (MIST) guidance without using radiolabeled compounds and chemically synthesized metabolite standards. Chem Res Toxicol 22(2): 311–322.

Walker D, Brady J, Dalvie D, Davis J, Dowty M, Duncan JN, Nedderman A, Obach RS & Wright P (2009) A holistic strategy for characterizing the safety of metabolites through drug discovery and development. Chem Res Toxicol 22(10): 1653–1662.

Walker GS, Atherton J, Bauman J, Kohl C, Lam W, Reily M, Lou Z & Mutlib A (2007) Determination of degradation pathways and kinetics of acyl glucuronides by NMR spectroscopy. Chem Res Toxicol 20(6): 876–886.

Walker GS, Bauman JN, Ryder TF, Smith EB, Spracklin DK & Obach RS (2014) Biosynthesis of drug metabolites and quantitation using NMR spectroscopy for use in pharmacologic and drug metabolism studies. Drug Metab Dispos 42(10): 1627–1639.

Walker GS, Ryder TF, Sharma R, Smith EB & Freund A (2011) Validation of isolated metabolites from drug metabolism studies as analytical standards by quantitative NMR. Drug Metab Dispos 39(3): 433–440.

Wang WW, Khetani SR, Krzyzewski S, Duignan DB & Obach RS (2010) Assessment of a micropatterned hepatocyte coculture system to generate major human excretory and circulating drug metabolites. Drug Metab Dispos 38(10): 1900–1905.

Waring MJ, Arrowsmith J, Leach AR, Leeson PD, Mandrell S, Owen RM, Pairaudeau G, Pennie WD, Pickett SD, Wang J, Wallace O & Weir A (2015) An analysis of the attrition of drug candidates from four major pharmaceutical companies. Nat Rev Drug Discov 14(7): 475–486.

Watkins PB (2011) Drug safety sciences and the bottleneck in drug development. Clin Pharmacol Ther 89(6): 788–790.

White RE, Evans DC, Hop CE, Moore DJ, Prakash C, Surapaneni S & Tse FL (2013) Radiolabeled mass-balance excretion and metabolism studies in laboratory animals: a commentary on why they are still necessary. Xenobiotica 43(2): 219–25; discussion 226–7.

Wienkers LC & Heath TG (2005) Predicting in vivo drug interactions from in vitro drug discovery data. Nat Rev Drug Discov 4(10): 825–833.

Williams DP (2006) Toxicophores: investigations in drug safety. Toxicology 226(1): 1–11.

Williams DP & Park BK (2003) Idiosyncratic toxicity: the role of toxicophores and bioactivation. Drug Discov Today 8(22): 1044–1050.

Williams JA, Hyland R, Jones BC, Smith DA, Hurst S, Goosen TC, Peterkin V, Koup JR & Ball SE (2004) Drug-drug interactions for UDP-glucuronosyltransferase substrates: a pharmacokinetic explanation for typically observed low exposure (AUCi/AUC) ratios. Drug Metab Dispos 32(11): 1201–1208.

Wrona M, Mauriala T, Bateman KP, Mortishire-Smith RJ & O'Connor D (2005) 'All-in-one' analysis for metabolite identification using liquid chromatography/hybrid quadrupole time-of-flight mass spectrometry with collision energy switching. Rapid Commun Mass Spectrom 19(18): 2597–2602.

Wu CY & Benet LZ (2005) Predicting drug disposition via application of BCS: transport/absorption/ elimination interplay and development of a biopharmaceutics drug disposition classification system. Pharm Res 22(1): 11–23.

Wurz GT, Kao CJ & DeGregorio MW (2014) Safety and efficacy of ospemifene for the treatment of dyspareunia associated with vulvar and vaginal atrophy due to menopause. Clin Interv Aging 9: 1939–1950.

Wurz GT, Soe LH & DeGregorio MW (2013) Ospemifene, vulvovaginal atrophy, and breast cancer. Maturitas 74(3): 220–225.

Xu L, Chen Y, Pan Y, Skiles GL & Shou M (2009) Prediction of human drug-drug interactions from time-dependent inactivation of CYP3A4 in primary hepatocytes using a population-based simulator. Drug Metab Dispos 37(12): 2330–2339.

Xu S, Zhu B, Teffera Y, Pan DE, Caldwell CG, Doss G, Stearns RA, Evans DC & Beconi MG (2005) Metabolic activation of fluoropyrrolidine dipeptidyl peptidase-IV inhibitors by rat liver microsomes. Drug Metab Dispos 33(1): 121–130.

Yamane N, Igarashi A, Kusama M, Maeda K, Ikeda T & Sugiyama Y (2013) Cost-effectiveness analysis of microdose clinical trials in drug development. Drug Metab Pharmacokinet 28(3): 187–195.

Yan Z & Caldwell GW (2004) Stable-isotope trapping and high-throughput screenings of reactive metabolites using the isotope MS signature. Anal Chem 76(23): 6835–6847.

Yan Z, Maher N, Torres R & Huebert N (2007) Use of a trapping agent for simultaneous capturing and high-throughput screening of both "soft" and "hard" reactive metabolites. Anal Chem 79(11): 4206–4214.

Yang Y, Grubb MF, Luk CE, Humphreys WG & Josephs JL (2011) Quantitative estimation of circulating metabolites without synthetic standards by ultra-high-performance liquid chromatography/high resolution accurate mass spectrometry in combination with UV correction. Rapid Commun Mass Spectrom 25(21): 3245–3251.

Yeo KR, Rostami-Hodjegan A & Tucker GT (2004) Abundance of cytochromes P450 in human liver: a meta-analysis. Br J Clin Pharmacol 57: 687–688.

Yeung CK, Fujioka Y, Hachad H, Levy RH & Isoherranen N (2011) Are circulating metabolites important in drug-drug interactions?: Quantitative analysis of risk prediction and inhibitory potency. Clin Pharmacol Ther 89(1): 105–113.

Young GC, Corless S, Felgate CC & Colthup PV (2008) Comparison of a 250 kV single-stage accelerator mass spectrometer with a 5 MV tandem accelerator mass spectrometer – fitness for purpose in bioanalysis. Rapid Commun Mass Spectrom 22(24): 4035–4042.

Young GC & Ellis WJ (2007) AMS in drug development at GSK. Nucl Instr and Meth in Phys Res B 259: 752–757.

Yu C, Chen CL, Gorycki FL & Neiss TG (2007) A rapid method for quantitatively estimating metabolites in human plasma in the absence of synthetic standards using a combination of liquid chromatography/mass spectrometry and radiometric detection. Rapid Commun Mass Spectrom 21(4): 497–502.

Yu H, Balani SK, Chen W, Cui D, He L, Humphreys WG, Mao J, Lai WG, Lee AJ, Lim HK, MacLauchlin C, Prakash C, Surapaneni S, Tse S, Upthagrove A, Walsky RL, Wen B & Zeng Z (2015) Contribution of metabolites to P450 inhibition-based drug-drug interactions: scholarship from the drug metabolism leadership group of the innovation and quality consortium metabolite group. Drug Metab Dispos 43(4): 620–630.

Yu H & Tweedie D (2013) A perspective on the contribution of metabolites to drug-drug interaction potential: the need to consider both circulating levels and inhibition potency. Drug Metab Dispos 41(3): 536–540.

Yu J, Ritchie TK, Mulgaonkar A & Ragueneau-Majlessi I (2014) Drug disposition and drug-drug interaction data in 2013 FDA new drug applications: a systematic review. Drug Metab Dispos 42(12): 1991–2001.

Yusof I, Shah F, Hashimoto T, Segall MD & Greene N (2014) Finding the rules for successful drug optimisation. Drug Discov Today 19(5): 680–687.

Zamek-Gliszczynski MJ, Abraham TL, Alberts JJ, Kulanthaivel P, Jackson KA, Chow KH, McCann DJ, Hu H, Anderson S, Furr NA, Barbuch RJ & Cassidy KC (2013) Pharmacokinetics, metabolism, and excretion of the glycogen synthase kinase-3 inhibitor LY2090314 in rats, dogs, and humans: a case study in rapid clearance by extensive metabolism with low circulating metabolite exposure. Drug Metab Dispos 41(4): 714–726.

Zamek-Gliszczynski MJ, Chu X, Polli JW, Paine MF & Galetin A (2014) Understanding the transport properties of metabolites: case studies and considerations for drug development. Drug Metab Dispos 42(4): 650–664.

Zamek-Gliszczynski MJ, Hoffmaster KA, Nezasa K, Tallman MN & Brouwer KL (2006a) Integration of hepatic drug transporters and phase II metabolizing enzymes: mechanisms of hepatic excretion of sulfate, glucuronide, and glutathione metabolites. Eur J Pharm Sci 27(5): 447–486.

Zamek-Gliszczynski MJ, Nezasa K, Tian X, Bridges AS, Lee K, Belinsky MG, Kruh GD & Brouwer KL (2006b) Evaluation of the role of multidrug resistance-associated protein (Mrp) 3 and Mrp4 in hepatic basolateral excretion of sulfate and glucuronide metabolites of acetaminophen, 4-methylumbelliferone, and harmol in Abcc3-/- and Abcc4-/- mice. J Pharmacol Exp Ther 319(3): 1485–1491.

Zanelli U, Caradonna NP, Hallifax D, Turlizzi E & Houston JB (2012) Comparison of cryopreserved HepaRG cells with cryopreserved human hepatocytes for prediction of clearance for 26 drugs. Drug Metab Dispos 40(1): 104–110.

Zhang D, Raghavan N, Wang L, Xue Y, Obermeier M, Chen S, Tao S, Zhang H, Cheng PT, Li W, Ramanathan R, Yang Z & Humphreys WG (2011) Plasma stability-dependent circulation of acyl glucuronide metabolites in humans: how circulating metabolite profiles of muraglitazar and peliglitazar can lead to misleading risk assessment. Drug Metab Dispos 39(1): 123–131.

Zhang F, Xue J, Shao J & Jia L (2012) Compilation of 222 drugs' plasma protein binding data and guidance for study designs. Drug Discov Today 17(9–10): 475–485.

Zhang H & Yang Y (2008) An algorithm for thorough background subtraction from high-resolution LC/MS data: application for detection of glutathione-trapped reactive metabolites. J Mass Spectrom 43(9): 1181–1190.

Zhang H, Zhang D & Ray K (2003) A software filter to remove interference ions from drug metabolites in accurate mass liquid chromatography/mass spectrometric analyses. J Mass Spectrom 38(10): 1110–1112.

Zhang H, Zhu M, Ray KL, Ma L & Zhang D (2008) Mass defect profiles of biological matrices and the general applicability of mass defect filtering for metabolite detection. Rapid Commun Mass Spectrom 22(13): 2082–2088.

Zhang N, Fountain ST, Bi H & Rossi DT (2000) Quantification and rapid metabolite identification in drug discovery using API time-of-flight LC/MS. Anal Chem 72(4): 800–806.

Zhang X, Bowles AC, Semon JA, Scruggs BA, Zhang S, Strong AL, Gimble JM & Bunnell BA (2014) Transplantation of autologous adipose stem cells lacks therapeutic efficacy in the experimental autoimmune encephalomyelitis model. PLoS One 9(1): e85007.

Zhao P, Zhang L, Grillo JA, Liu Q, Bullock JM, Moon YJ, Song P, Brar SS, Madabushi R, Wu TC, Booth BP, Rahman NA, Reynolds KS, Gil Berglund E, Lesko LJ & Huang SM (2011) Applications of physiologically based pharmacokinetic (PBPK) modeling and simulation during regulatory review. Clin Pharmacol Ther 89(2): 259–267.

Zhu M, Ma L, Zhang D, Ray K, Zhao W, Humphreys WG, Skiles G, Sanders M & Zhang H (2006) Detection and characterization of metabolites in biological matrices using mass defect filtering of liquid chromatography/high resolution mass spectrometry data. Drug Metab Dispos 34(10): 1722–1733.

Zhu M, Zhang D, Zhang H & Shyu WC (2009) Integrated strategies for assessment of metabolite exposure in humans during drug development: analytical challenges and clinical development considerations. Biopharm Drug Dispos 30(4): 163–184.

Zhu M, Zhang H & Humphreys WG (2011) Drug metabolite profiling and identification by high-resolution mass spectrometry. J Biol Chem 286(29): 25419–25425.

Zhu P, Ding W, Tong W, Ghosal A, Alton K & Chowdhury S (2009a) A retention-time-shift-tolerant background subtraction and noise reduction algorithm (BgS-NoRA) for extraction of drug metabolites in liquid chromatography/mass spectrometry data from biological matrices. Rapid Commun Mass Spectrom 23(11): 1563–1572.

Zhu P, Tong W, Alton K & Chowdhury S (2009b) An accurate-mass-based spectral-averaging isotope-pattern-filtering algorithm for extraction of drug metabolites possessing a distinct isotope pattern from LC-MS data. Anal Chem 81(14): 5910–5917.

Zientek MA & Youdim K (2015) Reaction phenotyping: advances in the experimental strategies used to characterize the contribution of drug-metabolizing enzymes. Drug Metab Dispos 43(1): 163–181.

# Original publications

I Makowiecki J, Tolonen A, Uusitalo J & Jalonen J (2001) Cone voltage and collision cell collision-induced dissociation study of triphenylethylenes of pharmaceutical interest. Rapid Commun Mass Spectrom 15(17): 1506–1513.

II Tolonen A, Koskimies P, Turpeinen M, Uusitalo J, Lammintausta R & Pelkonen O (2013) Ospemifene metabolism in humans *in vitro* and *in vivo*: metabolite identification, quantitation, and CYP assignment of major hydroxylations. Drug Metabol Drug Interact 28(3): 153–161.

III Turpeinen M, Uusitalo J, Lehtinen T, Kailajarvi M, Pelkonen O, Vuorinen J, Tapanainen P, Stjernschantz C, Lammintausta R & Scheinin M (2013) Effects of ospemifene on drug metabolism mediated by cytochrome P450 enzymes in humans *in vitro* and *in vivo*. Int J Mol Sci 14(7): 14064–14075.

IV Lehtinen T, Tolonen A, Turpeinen M, Uusitalo J, Vuorinen J, Lammintausta R, Pelkonen O & Scheinin M (2013) Effects of cytochrome P450 inhibitors and inducers on the metabolism and pharmacokinetics of ospemifene. Biopharm Drug Dispos 34(7): 387–395.

V Uusitalo J, Turpeinen M, Tolonen A, Koskimies P, Lammintausta R & Pelkonen O (2015) Metabolism and metabolite profiles *in vitro* and *in vivo* of ospemifene in humans and preclinical species. Drug Metabol Pers Ther 2016. DOI: 10.1515/dmdi-2015-0020. [Epub ahead of print.]



Original publications are not included in the electronic version of the dissertation.

Book orders:
Granum: Virtual book store
http://granum.uta.fi/granum/

UNIVERSITY OF OULU P.O. Box 8000 FI-90014 UNIVERSITY OF OULU FINLAND

# ACTA UNIVERSITATIS OULUENSIS



UNIVERSITY of OULU
OULUN YLIOPISTO

ISBN 978-952-62-1021-6 (Paperback)
ISBN 978-952-62-1022-3 (PDF)
ISSN 0355-3221 (Print)
ISSN 1796-2234 (Online)