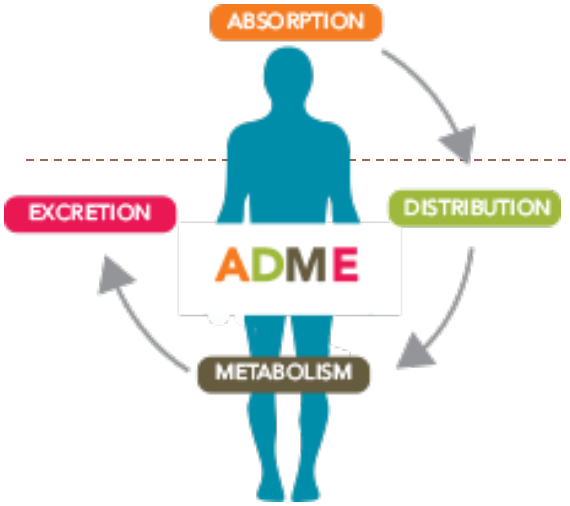
GLP - PLANET

ДОМ НПО ФАРМАЦИИ

Study of pharmacokinetic parameters in tests on the ADME panel

Kosman Vera M., PhD



ADME

Adsorption
Distribution
Metabolism
Excretion

In vivo – since 1950s the study of pharmacokinetics, bioavailability, tissue distribution, excretion, toxicity on two species of animals (rodents and non-rodents) before the study of pharmacokinetics in healthy volunteers (1 phase CI) is necessary by regulation documents.

In vitro – since the 1990s with the development of cellular and molecular biology, high-performance and miniaturizing technologies in the early 21 century, tests outside living systems began to be introduced, allowing to reduce the volume of further studies in vivo, based on the selection of only the most promising candidates. Considerable attention is paid to extrapolation of in vitro/in vivo data.

Normative documents

The system of tests constituting the so-called ADME panel is aimed at studying biotransformation and drug transporters in vitro [1-4].

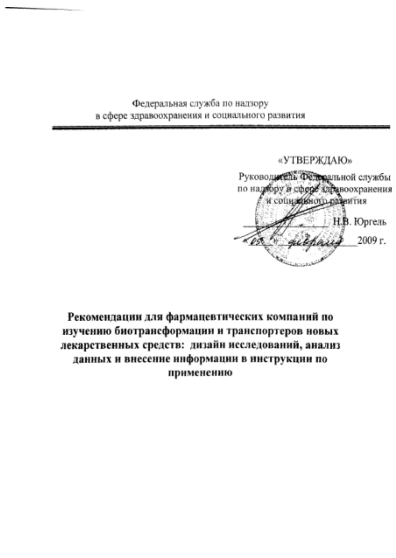
In vitro studies serve as a screening method to assess the role of biotransformation in drug pharmacokinetics, should precede appropriate in vivo studies in humans [1-2], and are necessary to prepare an drug registration file for medical use according to current requirements [3-5].

1. Руководство по экспертизе лекарственных средств. Т.3. – М.: Полиграф-Плюс, 2014. 344 с. Guidelines for the examination of medicines. T.3. - M.: Polygraph-Plus, 2014. 344 p.
2. Рекомендации для фармацевтических компаний по изучению биотрансформации и транспортеров новых лекарственных средств: дизайн исследований, анализ данных и внесение информации в инструкцию по применению./ Сост. Сычев Д.А., Кукес В.Г. – М. Федеральная служба по надзору в сфере здравоохранения и социального развития. 2009. 32 с. Recommendations for pharmaceutical companies for the study of biotransformation and transporters of new drugs: research design, data analysis and the introduction of information in the instructions for use./Comp. Sychev D.A., Kukes V.G. - M. Federal Service for Supervision in the Field of Health and Social Development. 2009. 32 p.
3. Guidance for Industry. Drug Metabolism/Drug Interaction Studies in the Drug Development Process: Studies In Vitro. Rockville, MD, U.S. Department of Health and Human Services, FDA, Center for Drug Evaluation and Research, Center For Biologics Evaluation and Research, 2007.
4. Guideline on the Investigation of Drug Interactions. CPMP/EWP/560/95/Rev. 1 – Corr. EMEA. Committee for Human Medicinal Products (CHMP). 2007.
5. Решение Совета ЕЭК №78 от 03.12.16 «О Правилах регистрации и экспертизы лекарственных средств для медицинского применения». Decision of the ECE Council No. 78 of 03.12.16 "On the Rules for the Registration and Examination of Medicines for Medical Use."

Documents

Recommendations for pharmaceutical companies for the study of biotransformation and transporters of new drugs: research design, data analysis and the introduction of information in the instructions for use./Comp. Sychev D.A., Kukes V.G. - M. Federal Service for Supervision in the Field of Health and Social Development. 2009. 32 p.

Chapter 5. Methodological Recommendations for the Study of Biotransformation and Transporters of New Drugs: Study Design, Data Analysis.//V kn.: Guide to Drug Examination. T.3. - M.: Polygraph-Plus, 2014. - C.68-92.

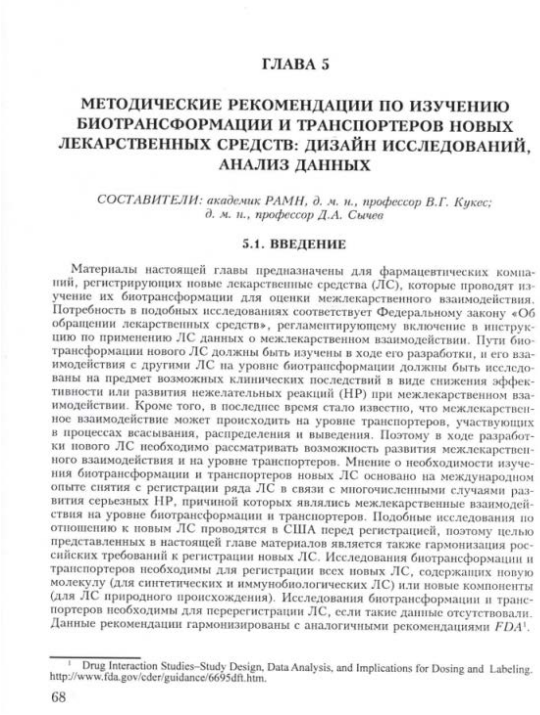


Федеральная служба по надзору
в сфере здравоохранения и социального развития

«УТВЕРЖДАЮ»
Руководитель Федеральной службы
по надзору в сфере здравоохранения
и социального развития
Н.В. Юргель
2009 г.

Рекомендации для фармацевтических компаний по изучению биотрансформации и транспортеров новых лекарственных средств: дизайн исследований, анализ данных и внесение информации в инструкции по применению

Москва 2009



ГЛАВА 5

МЕТОДИЧЕСКИЕ РЕКОМЕНДАЦИИ ПО ИЗУЧЕНИЮ БИОТРАНСФОРМАЦИИ И ТРАНСПОРТЕРОВ НОВЫХ ЛЕКАРСТВЕННЫХ СРЕДСТВ: ДИЗАЙН ИССЛЕДОВАНИЙ, АНАЛИЗ ДАННЫХ

СОСТАВИТЕЛИ: академик РАМН, д. м. н., профессор В.Г. Кукес; д. м. н., профессор Д.А. Сычев

5.1. ВВЕДЕНИЕ

Материалы настоящей главы предназначены для фармацевтических компаний, регистрирующих новые лекарственные средства (ЛС), которые проводят изучение их биотрансформации для оценки межлекарственного взаимодействия. Потребность в подобных исследованиях соответствует Федеральному закону «Об обращении лекарственных средств», регламентирующему включение в инструкцию по применению ЛС данных о межлекарственном взаимодействии. Пути биотрансформации нового ЛС должны быть изучены в ходе его разработки, и его взаимодействия с другими ЛС на уровне биотрансформации должны быть исследованы на предмет возможных клинических последствий в виде снижения эффективности или развития нежелательных реакций (НР) при межлекарственном взаимодействии. Кроме того, в последнее время стало известно, что межлекарственное взаимодействие может происходить на уровне транспортеров, участвующих в процессах всасывания, распределения и выведения. Поэтому в ходе разработки нового ЛС необходимо рассматривать возможность развития межлекарственного взаимодействия и на уровне транспортеров. Мнение о необходимости изучения биотрансформации и транспортеров новых ЛС основано на международном опыте снятия с регистрации ряда ЛС в связи с многочисленными случаями развития серьезных НР, причиной которых являлись межлекарственные взаимодействия на уровне биотрансформации и транспортеров. Подобные исследования по отношению к новым ЛС проводятся в США перед регистрацией, поэтому целью представленных в настоящей главе материалов является также гармонизация российских требований к регистрации новых ЛС. Исследования биотрансформации и транспортеров необходимы для регистрации всех новых ЛС, содержащих новую молекулу (для синтетических и иммунобиологических ЛС) или новые компоненты (для ЛС природного происхождения). Исследования биотрансформации и транспортеров необходимы для перерегистрации ЛС, если такие данные отсутствовали. Данные рекомендации гармонизированы с аналогичными рекомендациями *FDA*[1].

[1] Drug Interaction Studies–Study Design, Data Analysis, and Implications for Dosing and Labeling. http://www.fda.gov/cder/guidance/6695dft.htm.

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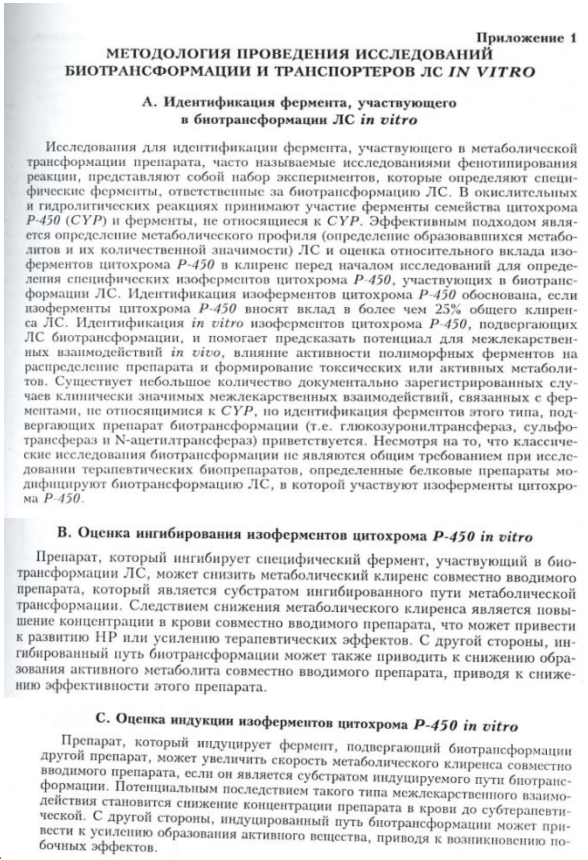


Приложение 1

МЕТОДОЛОГИЯ ПРОВЕДЕНИЯ ИССЛЕДОВАНИЙ БИОТРАНСФОРМАЦИИ И ТРАНСПОРТЕРОВ ЛС *IN VITRO*

А. Идентификация фермента, участвующего в биотрансформации ЛС *in vitro*

Исследования для идентификации фермента, участвующего в метаболической трансформации препарата, часто называемые исследованиями фенотипирования реакции, представляют собой набор экспериментов, которые определяют специфические ферменты, ответственные за биотрансформацию ЛС. В окислительных и гидролитических реакциях принимают участие ферменты семейства цитохрома *P-450* (*CYP*) и ферменты, не относящиеся к *CYP*. Эффективным подходом является определение метаболического профиля (определение образовавшихся метаболитов и их количественной значимости) ЛС и оценка относительного вклада изоферментов цитохрома *P-450* в клиренс перед началом исследований для определения специфических изоферментов цитохрома *P-450*, участвующих в биотрансформации ЛС. Идентификация изоферментов цитохрома *P-450* обоснована, если изоферменты цитохрома *P-450* вносят вклад в более чем 25% общего клиренса ЛС. Идентификация *in vitro* изоферментов цитохрома *P-450*, подвергающих ЛС биотрансформации, и помогает предсказать потенциал для межлекарственных взаимодействий *in vivo*, влияние активности полиморфных ферментов на распределение препарата и формирование токсических или активных метаболитов. Существует небольшое количество документально зарегистрированных случаев клинически значимых межлекарственных взаимодействий, связанных с ферментами, не относящимися к *CYP*, но идентификация ферментов этого типа, подвергающих препарат биотрансформации (т.е. глюкозуронилтрансфераз, сульфотрансфераз и N-ацетилтрансфераз) приветствуется. Несмотря на то, что классические исследования биотрансформации не являются общим требованием при исследовании терапевтических биопрепаратов, определенные белковые препараты модифицируют биотрансформацию ЛС, в которой участвуют изоферменты цитохрома *P-450*.

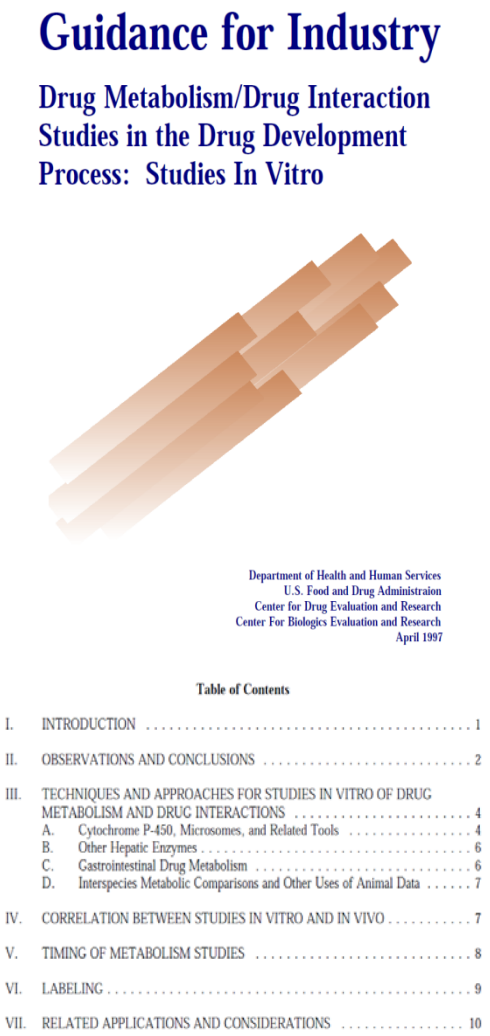
В. Оценка ингибирования изоферментов цитохрома *P-450 in vitro*

Препарат, который ингибирует специфический фермент, участвующий в биотрансформации ЛС, может снизить метаболический клиренс совместно вводимого препарата, который является субстратом ингибированного пути метаболической трансформации. Следствием снижения метаболического клиренса является повышение концентрации в крови совместно вводимого препарата, что может привести к развитию НР или усилению терапевтических эффектов. С другой стороны, ингибированный путь биотрансформации может также приводить к снижению образования активного метаболита совместно вводимого препарата, приводя к снижению эффективности этого препарата.

С. Оценка индукции изоферментов цитохрома *P-450 in vitro*

Препарат, который индуцирует фермент, подвергающий биотрансформации другой препарат, может увеличить скорость метаболического клиренса совместно вводимого препарата, если он является субстратом индуцируемого пути биотрансформации. Потенциальным последствием такого типа межлекарственного взаимодействия становится снижение концентрации препарата в крови до субтерапевтической. С другой стороны, индуцированный путь биотрансформации может привести к усилению образования активного вещества, приводя к возникновению побочных эффектов.

Documents



Guidance for Industry

Drug Metabolism/Drug Interaction Studies in the Drug Development Process: Studies In Vitro

Department of Health and Human Services
U.S. Food and Drug Administraion
Center for Drug Evaluation and Research
Center For Biologics Evaluation and Research
April 1997

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- Guidance for Industry. Drug Metabolism/Drug Interaction Studies in the Drug Development Process: Studies In Vitro. Rockville, MD, U.S. Department of Health and Human Services, FDA, Center for Drug Evaluation and Research, Center For Biologics Evaluation and Research, 2007.
- Guideline on the Investigation of Drug Interactions. CPMP/EWP/560/95/Rev. 1 – Corr. EMEA. Committee for Human Medicinal Products (CHMP). 2007.

EUROPEAN MEDICINES AGENCY
SCIENCE MEDICINES HEALTH

21 June 2012
CPMP/EWP/560/95/Rev. 1 Corr. 2**
Committee for Human Medicinal Products (CHMP)

Guideline on the investigation of drug interactions

Discussion in the Efficacy Working Party (EWP)	June/October 1996 February 1997
Transmission to the CPMP	March 1997
Transmission to interested parties	March 1997
Deadline for comments	September 1997
Re-submission to the EWP	December 1997
Approval by the CPMP	December 1997
Date for coming into operation	June 1998
Draft Rev. 1 Agreed by the EWP	April 2010
Adoption Rev. 1 by CHMP for release for consultation	22 April 2010
End of consultation Rev. 1 (deadline for comments)	31 October 2010
Agreed by Pharmacokinetics Working Party	February 2012
Adopted by CHMP	21 June 2012
Date for coming into effect	1 January 2013

This guideline replaces guideline CPMP/EWP/560/95.

Keywords	*Interaction, guideline, metabolism, inhibition, induction, transport, enzyme, transport protein, transporter, absorption, food, distribution, PBPK, herbal, SmPC*

* The correction concerns section 5.3.4.1 (p 26) and the corresponding decision tree no. 6 (p 61) to read "if the observed Ki value is lower or equal to /.../"; Appendix VII, Table 5 to read "See section 5.4.2".* Decision tree 4.

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Documents. Guideline on the Investigation of Drug Interactions

Guideline on the investigation of drug interactions

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5.2.1. Absorption

B. Interactions affecting intestinal active transport

It is recommended that the involvement of transporters in drug absorption is evaluated *in vitro* in Caco-2 cells. To evaluate the importance of active transport for drug absorption, the permeability of the investigational drug should be taken into account. If the *in vitro* transport and permeability data indicate that active intestinal transport may affect the bioavailability of the new drug, attempts should be made to identify the transporter involved *in vitro*. Detailed recommendations on how to study intestinal transporter involvement and to determine the apparent permeability constant *in vitro* is given in appendices II and III.

5.2.3. Metabolism

In vitro metabolism studies should generally be performed before starting phase I to identify the main metabolites formed *in vitro*. These studies provide information necessary for the extrapolation of preclinical safety data to man and also allow for early screening of main metabolites found *in vitro* for target pharmacological activity.

In vitro studies should also be performed to identify candidate enzymes responsible for the main metabolic pathways of the parent drug. Guidance on the *in vitro* investigations of which enzymes are involved in the metabolism is given in Appendix IV.

5.2.4. Active uptake and secretion in drug elimination

Information on transporters involved in major elimination processes should be gained as early as possible during drug development. The need for data at different phases is driven by the predicted magnitude of the exposure increase if the transporter is inhibited and the clinical consequences of such an increase. *In vitro* data may be sufficient before phase III provided use of potentially significantly interacting drugs may be restricted in the study protocol.

In vitro studies usually compose the first steps of the identification process. The *in vitro* studies are further described in Appendix III. The transporters selected for *in vitro* investigation should be based on available expression data of the transporter in the eliminating organ and, if possible, whether uptake or efflux transporters or a combination are expected to be involved and be rate limiting for the elimination process.

Documents. Guideline on the Investigation of Drug Interactions

5.3. Effects of the investigational drug on the pharmacokinetics of other drugs

5.3.3. Metabolism

5.3.3.1 Enzyme inhibition

A. *In vitro* enzyme inhibition studies

In vitro studies should be performed to investigate whether the investigational drug inhibits the cytochrome P450 enzymes most commonly involved in drug metabolism. These presently include CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A. In the future, more clinically important drug metabolising enzymes may be known and included in this list. In addition, it is recommended to study inhibition of UGTs known to be involved in drug interactions, including UGT1A1 and UGT2B7, if one of the major elimination pathways of the investigational drug is direct glucuronidation. Likewise, if the investigational drug is mainly metabolised by an enzyme not listed above, it is recommended to study the inhibitory effect on that specific enzyme if feasible. It is also recommended to investigate the enzyme inhibitory effect of major metabolites. More information on this is found in the end of this subsection.

An *in vitro* inhibition study could be performed using human liver microsomes, hepatocytes, or other cells expressing the investigated enzyme. The enzyme activity is monitored by investigating the metabolism of a specific marker substrate (Table 4, Appendix VII) under linear substrate metabolism conditions. CYP3A4 inhibition should be investigated using both midazolam and testosterone as subsrates. The effect of a range of investigational drug concentrations are investigated and Ki (the inhibition constant i.e. dissociation constant of the inhibitor from the enzyme-inhibitor complex) is determined. The study should include an investigation of whether pre-incubation with the investigational drug alters the inhibitory potential of the drug. If the pre-incubation affects the potency, more detailed investigations are needed (see below). If the investigational drug is metabolised by the enzymes present in the incubation, the marker substrate should, if possible, have a markedly faster metabolism rate than the investigational drug to minimize the influence of investigational drug metabolism (decreasing concentrations) on the Ki estimation. If this is not possible, the concentrations of the investigational drug need to be monitored and/or the degradation taken into account in the calculations. Known strong inhibitors should be included as positive controls in the study, their Ki determined and compared to literature/reference in house values. The concentration range of the investigational drug should be sufficiently high for detecting clinically relevant inhibition and depends on the potential site of enzyme inhibition, mode of administration and formulation as well as systemic exposure. It is recommended to use the estimated or determined unbound drug concentration in the *in vitro* system. In situations where it is important to have a precise value on fu_{mic} (unbound microsomal fraction), such as estimations of inhibition or induction potential not followed by an *in vivo* study, determining the fraction (experimentally) is recommended. This also applies if there are reasons to believe that the free inhibitor concentration is markedly lower than the total concentration in the incubation, i.e. if the substance binds covalently to proteins or may adsorb to the walls of the test tube.

5.3.4. Transport

5.3.4.1. Inhibition of transport proteins

In vitro inhibition studies are recommended to investigate whether the investigational drug inhibits any of the transporters known to be involved in clinically relevant *in vivo* drug interactions. Presently, these include P-glycoprotein/MDR1 (ABCB1), OATP1B1 (SLCO1B1), OATP1B3 (SLCO1B3), OCT2 (SLC22A2), OAT1 (SLC22A6), OAT3 (SLC22A8) and BCRP (ABCG2). Investigations of the inhibitory effect on OCT1 (SLC22A1), MATE1 (SLC47A1) and MATE2 (SLC47A) could also be considered. Inhibition of the transporter BSEP (ABCB11) should also preferably be investigated. If *in vitro* studies indicate BSEP inhibition adequate biochemical monitoring including serum bile salts is recommended during drug development. *In vitro* data on transporter inhibition should preferably be available before initiating phase III. The knowledge about transporters and their *in vivo* importance is evolving quickly. The choice of transporters investigated should be driven by scientific evidence, and transporters may be added to or removed from the list as science develops. In addition to the listed transporters, there may also be a need to investigate effects on other transporters to clarify the mechanism of an unexpected interaction observed *in vivo*. As science is rapidly evolving in this field, no lists on *in vitro* and *in vivo* substrates are presented as such lists may need frequent updates. The substrates/probe drugs chosen should be based on the current scientific literature.

Appendix II

***In vitro* investigations of involvement of transporters in drug absorption and determinations of the apparent permeability constant**

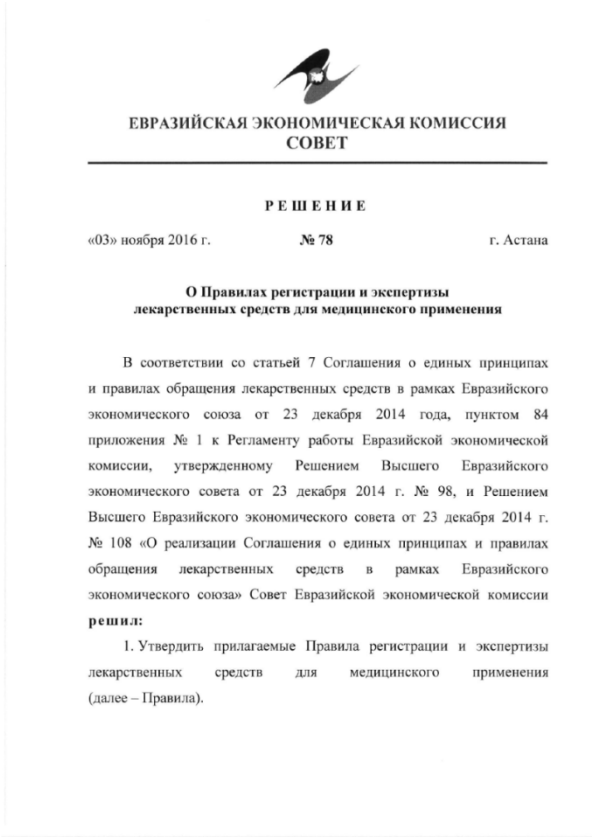
The *in vitro* investigation of transporter involvement at an intestinal level usually begins with Caco-2 experiments. In these cells, the intestinal permeability may also be qualitatively assessed. Several transporters may be present in the Caco-2 cells. Other *in vitro* systems may be used as well to investigate transporter involvement and may have advantages depending on if specific inhibitors are available. This appendix focuses on Caco-2 cell studies, while other *in vitro* systems are discussed in Appendix III.

Appendix III

***In vitro* studies identifying transporters involved in drug disposition**

The identification of which transporter is involved in an identified uptake or efflux process affecting drug disposition may be done *in vitro* through transport studies intended to isolate the effect of a specific transporter.

Documents



ЕВРАЗИЙСКАЯ ЭКОНОМИЧЕСКАЯ КОМИССИЯ
СОВЕТ

Р Е Ш Е Н И Е

«03» ноября 2016 г. **№ 78** г. Астана

О Правилах регистрации и экспертизы лекарственных средств для медицинского применения

В соответствии со статьей 7 Соглашения о единых принципах и правилах обращения лекарственных средств в рамках Евразийского экономического союза от 23 декабря 2014 года, пунктом 84 приложения № 1 к Регламенту работы Евразийской экономической комиссии, утвержденному Решением Высшего Евразийского экономического совета от 23 декабря 2014 г. № 98, и Решением Высшего Евразийского экономического совета от 23 декабря 2014 г. № 108 «О реализации Соглашения о единых принципах и правилах обращения лекарственных средств в рамках Евразийского экономического союза» Совет Евразийской экономической комиссии **решил:**

1. Утвердить прилагаемые Правила регистрации и экспертизы лекарственных средств для медицинского применения (далее – Правила).

4.2.2. Фармакокинетика.

Документы регистрационного досье по фармакокинетическим исследованиям включают результаты анализа всех процессов, происходящих с активным веществом и его метаболитами в живом организме, и охватывают изучение абсорбции, распределения, биотрансформации и выведения активного вещества и его метаболитов.

Исследование каждого из этапов (абсорбции, распределения, биотрансформации и выведения) может выполняться как с помощью физических, химических или биологических методов, так и посредством изучения фактической фармакодинамической активности самого активного вещества.

Информация о распределении и выведении активного вещества из организма является необходимой во всех случаях, когда данные о распределении и выведении активного вещества являются обязательными для определения дозы лекарственного препарата для человека, а также для химиотерапевтических веществ (антибиотиков и др.) и веществ, использование которых зависит от их нефармакодинамических эффектов (например, диагностические препараты и др.).

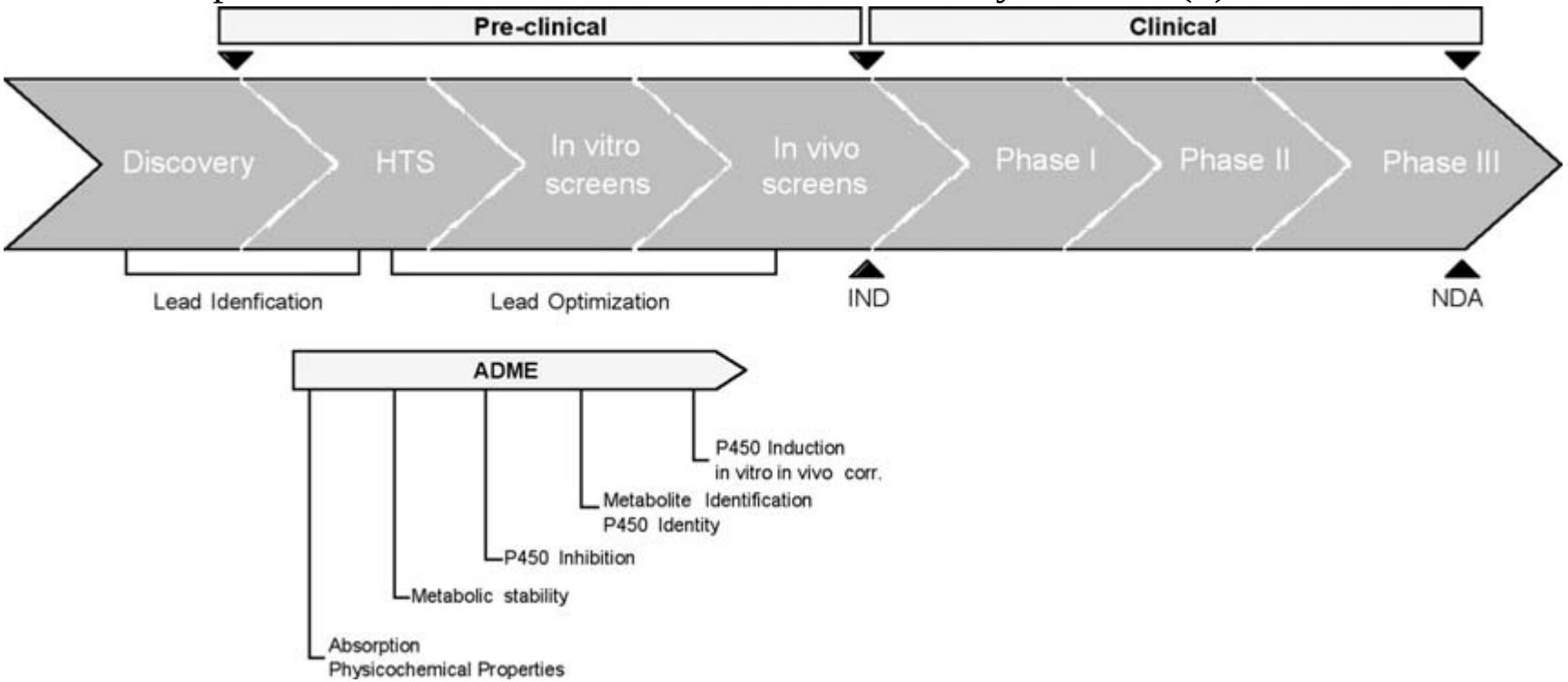
Исследования in vitro целесообразно проводить с использованием тест-систем, полученных от человека, нежели с использованием тест-системам животного происхождения (например, исследование связывания активного вещества с белками, метаболизма, лекарственных взаимодействий).

5.3.2. Отчеты о фармакокинетических исследованиях с использованием биоматериалов человека.

К биоматериалам, полученным от человека, относят белки, клетки, ткани и связанные с ними материалы, полученные от человека, которые используются при проведении исследований in vitro или in vivo для оценки фармакокинетических свойств активных веществ. В регистрационном досье лекарственного препарата необходимо представить отчеты об исследовании связывания активного вещества с белками плазмы, метаболизма в печени и взаимодействия активного вещества, а также исследования с использованием других биоматериалов, полученных от человека.

Publications

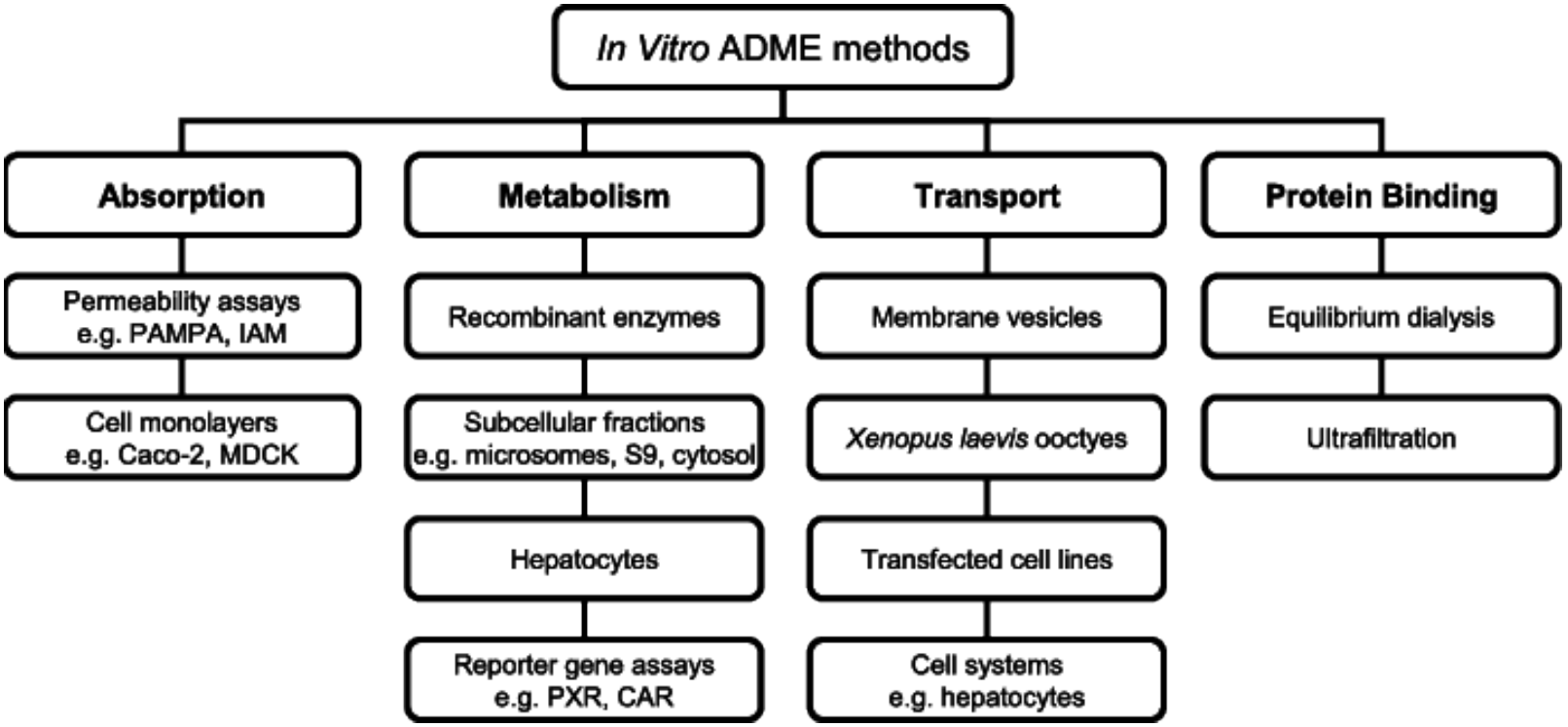
A. Lahoz, L. Gombau, M. T. Donato, J. V Castell, M. J. Gómez-Lechón
In Vitro ADME Medium/High-Throughput Screening in Drug Preclinical Development. Mini-Reviews in Medicinal Chemistry, 2006, 6 (9): 1053-1062.



Schematic representation of drug development process, depicting the different types of ADME studies that could be perform at the various stages. Investigational New Drug (IND). New Drug Application (NDA).

Publications

Smith N.,F., Raynaud F., Workman P. The application of cassette dosing for pharmacokinetic screening in small-molecule cancer drug discovery. Molecular Cancer Therapeutics. 2007; 6(2):428-40



Summary of in vitro ADME methods

Publications

Wan H. What ADME tests should be conducted for preclinical studies? ADMET & DMPK.2013; 1(3) :19-28.

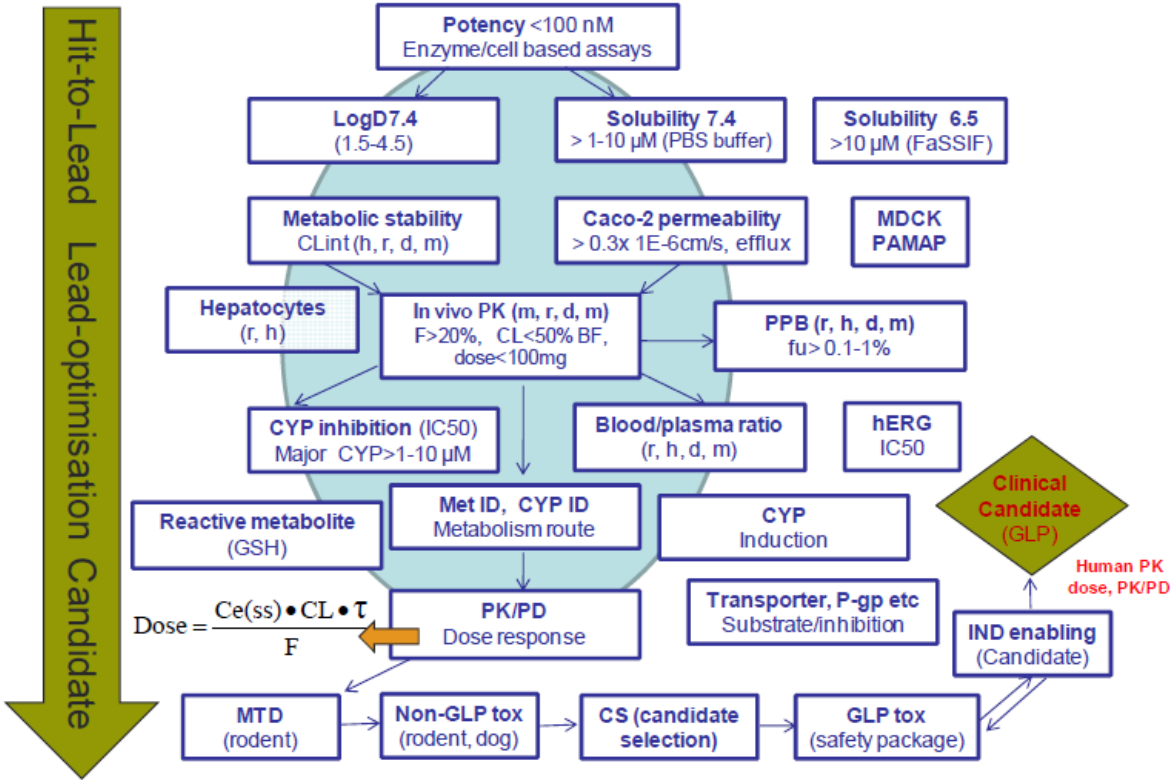


Figure 1: Typical ADME/PK screening cascade

Drug metabolism and pharmacokinetics (DMPK) services

- Physicochemical screening
  - Aqueous solubility (Thermodynamic and Kinetic method)
  - Log P (Octanol/water)
  - Log D (Octanol/PBS)
- Permeability
  - PAMPA
  - CaCo – 2
  - MDCK (wild type)
- Protein Binding
  - Plasma Protein Binding (Equilibrium Dialysis)
- Drug Metabolism
  - Hepatocyte Stability
  - Microsomal Stability
  - Plasma Stability
- Drug-Drug interaction
  - CYP inhibition assays (Fluorescent and LCMS/MS based)
- Others
  - P-Glycoprotein Substrate
  - P-Glycoprotein Inhibition
  - Preliminary BCS Permeability (non-GLP)
  - Metabolite Identification (without structure elucidation)

Creative Biolabs

cyprotex
AN EVOTEC COMPANY

IRBM

IONTOX
Innovative In Vitro Solutions

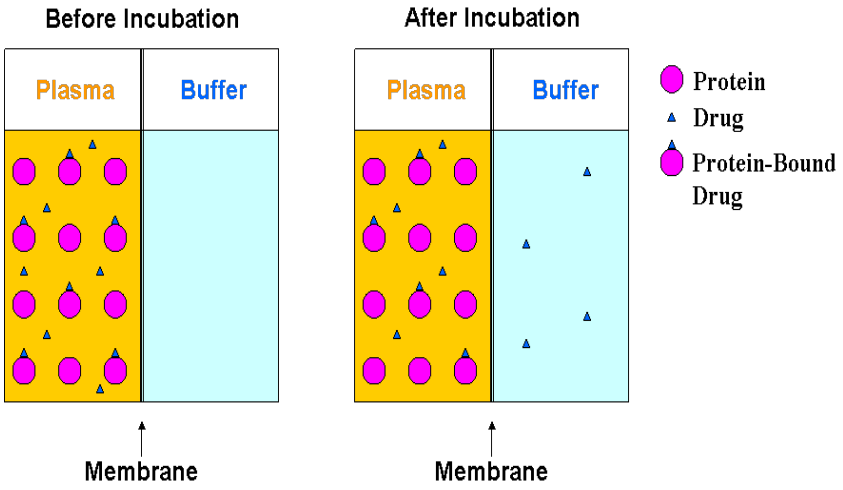
eurofins | DISCOVERY

Minimum tests panel

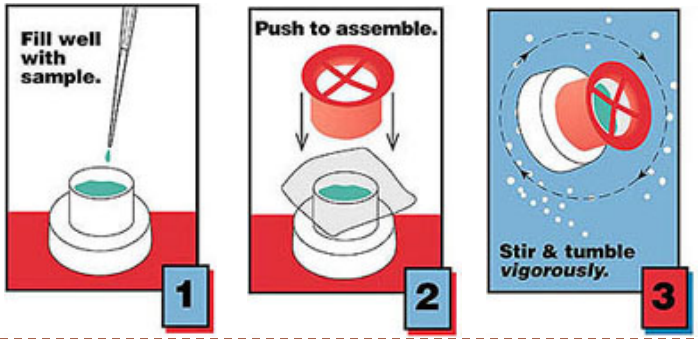
- Binding to blood plasma proteins
- Stability in liver microsomes
- Cell permeability assessment (Caco2 model)

Binding to blood plasma proteins

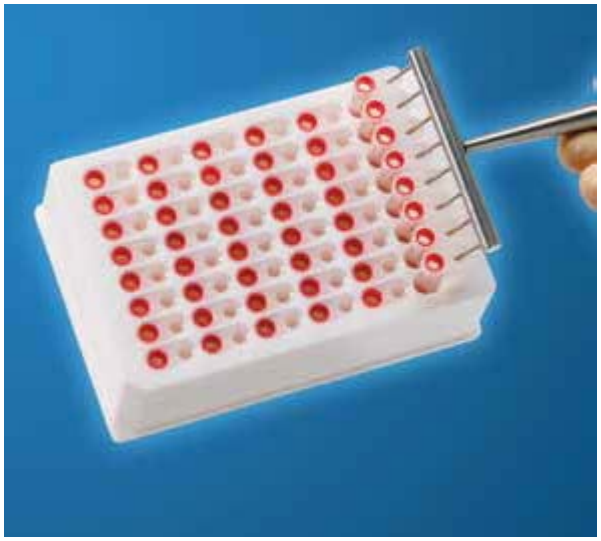
- Dialysis method
- Semi-permeable membrane with pore diameter 7-8 kDa
- % free unchanged analyte (in acceptor medium, HPLC)
- % bound analyte = 100% -% free analyte



QuixSep® MICRO DIALYZER

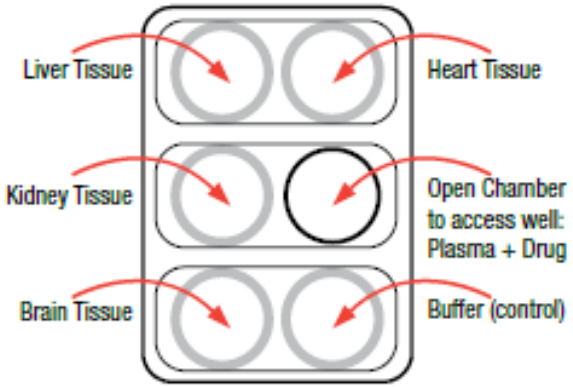


The automated multichannel systems Thermo Scientific Matrix PlateMate Automation



Advantages:
No/minimized sample dilution
Parallel testing for a large number of samples
Reduce test time by reducing dialysis equilibrium time

Competition RED Device



The following charts compare results of the *in vitro* and *in vivo* studies:

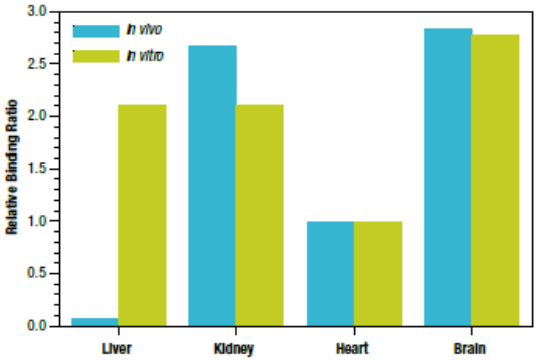


Figure 7. Comparison of *in vitro* and *in vivo* tissue and plasma-binding ratios of propranolol in rat. Low propanolol presence in liver tissue *in vivo* is mainly due to high hepatic metabolic activities.

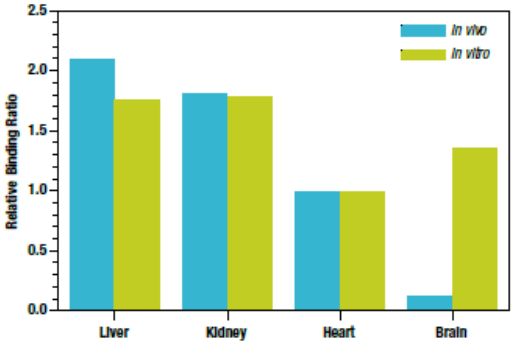


Figure 8. Comparison of *in-vitro* and *in-vivo* tissue/plasma binding ratios of Taxol in rat. Low Taxol presence in brain tissue *in vivo* indicates the blood-brain barrier inhibits movement of Taxol to the brain.

The Thermo Fisher Scientific RED Device Systems Brochure. Thermo Fisher Scientific Inc. 2012. 8 p.

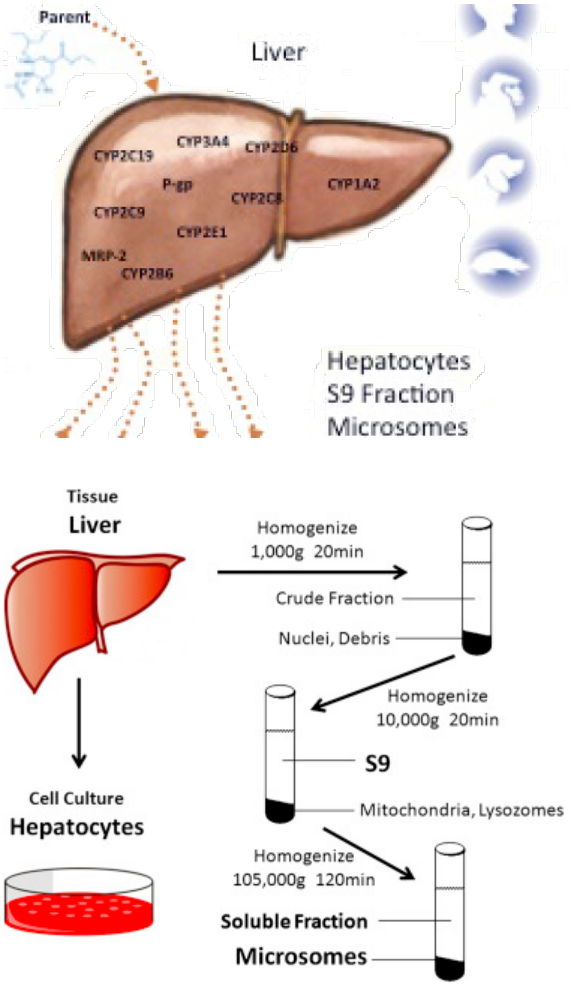
Stability in liver microsomes

Evaluation of stability in the microsomal fraction of the liver is a primary test of the ADME panel, since liver microsomes are a subcellular fraction containing basic metabolic enzymes such as monooxygenases.

The essence of the test consists in incubating the test substance with liver microsomes in the presence of a metabolism phase I cofactor - nicotinamide adenine dinucleotide phosphate (NADP, NADPH) with subsequent assessment of the change in the content of the analyzed compound (by HPLC) at several time points.

Human liver microsomes (Microsomes from liver, pooled, from human, M0442, Sigma, Germany or the like) or other biological species may be used.

In parallel, a control substance with known activity (positive control) is tested, as which verapamil hydrochloride is often used.

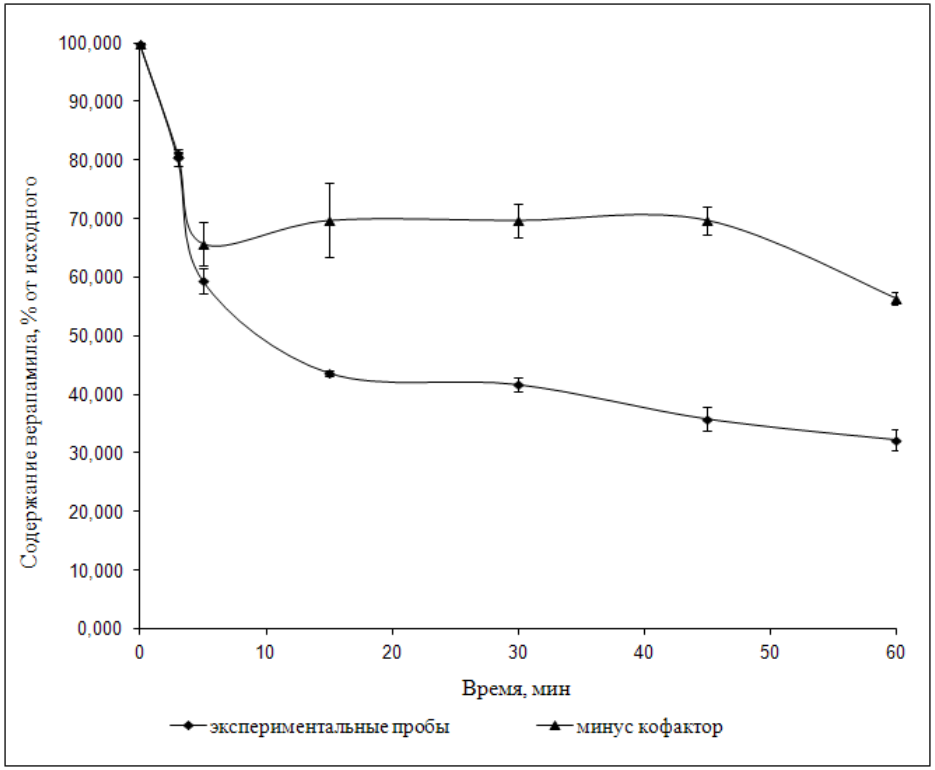


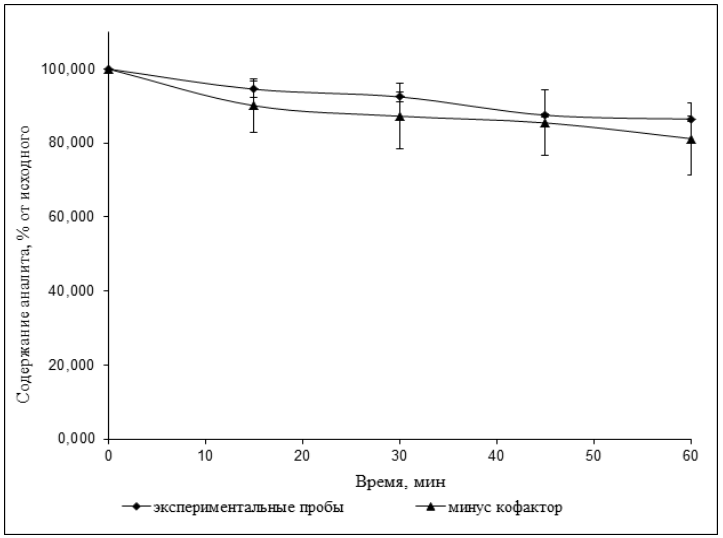
Stability in liver microsomes

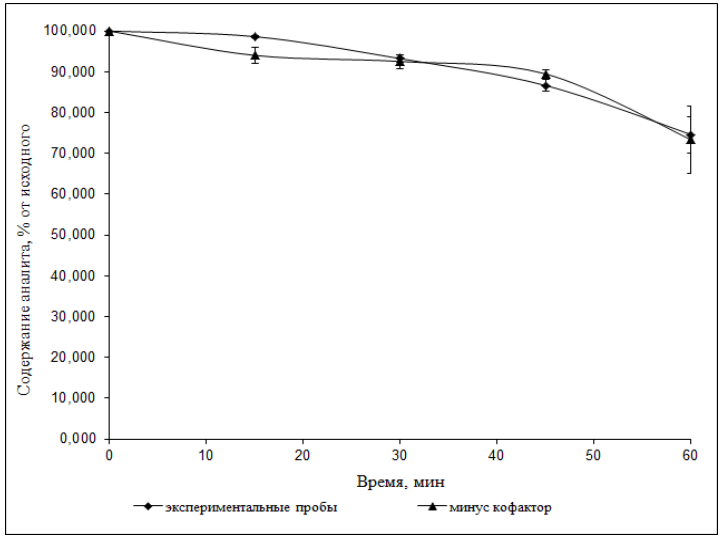
Exemplary Scheme of the Microsomal Stability Evaluation Experiment
test tubes 1,2 - experimental, 3,4 - minus cofactor, 5, 6 - form-samples; the number of parallel tubes can be changed.

Group of samples; the brought substance	N of tube	Volumes of solutions introduced before preincubation, ml			Volumes of solutions introduced after preincubation, ml	
		Microsomes suspension	NADPhH solution	Buffer solution with pH 7,4	Analyte solution	Buffer solution with c pH 7,4
1; R1 (ontrol compound - verapamyl hydrochloride)	1	0,025	0,1	0,35	0,025	0
	2	0,025	0,1	0,35	0,025	0
	3	0,025	0	0,45	0,025	0
	4	0,025	0	0,45	0,025	0
	5	0,025	0,1	0,35	0	0,025
	6	0,025	0,1	0,35	0	0,025
2; T1 (test compound)	1	0,025	0,1	0,35	0,025	0
	2	0,025	0,1	0,35	0,025	0
	3	0,025	0	0,45	0,025	0
	4	0,025	0	0,45	0,025	0
	5	0,025	0,1	0,35	0	0,025
	6	0,025	0,1	0,35	0	0,025

Stability in liver microsomes







Stability in liver microsomes

Test results are used to calculate half-life ($T_{1/2}$), internal clearance (Clint), remaining amount of analyte (% of initial).

Based on the obtained areas of chromatographic peaks at each time point, the amount of analyte (C) in% of the original is calculated, taking the peak area at the first time point (0 min) as 100%.

Concentration values of the analysed compounds (C,% of the initial) are logarithmized and the obtained lnC values for each compound are plotted against time to obtain linear dependencies of the form y = a * x + b.

Coefficients a of obtained dependencies are used for calculation of half-life of $T_{1/2}$ by formula (1):

$$\mathbf{T_{1/2}=0,693/|a|} \quad (1),$$

where a is the coefficient a of the corresponding linear equation of the form y = a * x + b.

Internal clearance (Clint, μg/min/mg liver microsome) was calculated by formula (2):

$$\mathbf{Clint = V \cdot 0,693 / m \cdot T_{1/2}} \quad (2),$$

where V is the volume of the reaction mixture, μl (500 μl);

m is the weight of the protein in the liver microsome in the reaction, mg (0.5 mg);

$T_{1/2}$ - half-life, min.

Stability in liver microsomes

Based on our experimental experience, $T_{1/2}$ values can range from 13-14 minutes to more than 480 minutes.

According to [Microsomal stability assay. https ://www.cyprotex.com/admepk/in-vitro-metabolism/microsomal-stability/.] drugs can be classified according to the level of internal clearance.

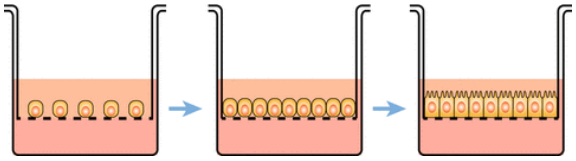
Type	Internal clearance (Clint, µg/min/mg liver microsome)				
	Human	Monkey	Dog	Rat	Mice
Low cliarance	< 8,6	< 12,5	< 5,3	< 13,2	< 8,8
High clearance	> 47,0	> 67,8	> 28,9	> 71,9	> 48,0

Cell permeability assessment (Caco2 model)

1. Cell culture and production of a cellular monolayer

Cells of the Caco-2 line (human colorectal adenocarcinoma, Vertebrate Cell Culture Collection, FSBUN Institute of Cytology of the Russian Academy of Sciences) are cultured in plastic vials in a special medium. Subcultivation (over) of cells was carried out when they reached 80% monolayer 1-2 times a week.

The growth of the dense cell monolayer Caco-2 was carried out on permeable inserts. In order to synchronize the differentiation process and to form the monolayer as quickly as possible, the cells must be scattered in a very high concentration. After scattering, the cells were cultured for 21-25 days. The transepithelial electrical resistance (TPP) was measured periodically during cell culture with Millicell ® ERS-2 Volt-Ohm meter to confirm cell monolayer formation and evaluation**.**



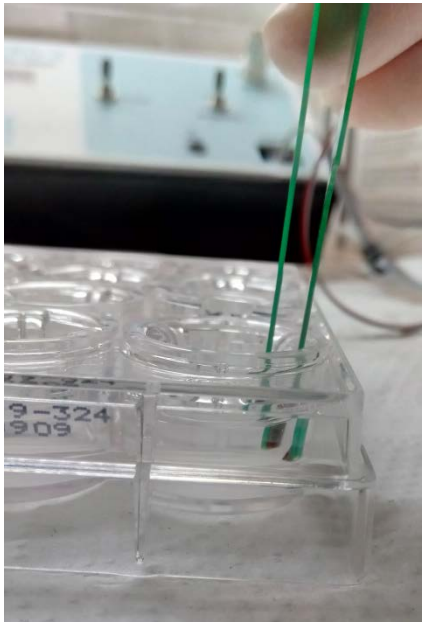
Cell permeability assessment (Caco2 model)

2. Evaluation of the damaging effect of test objects on the integrity of the cellular monolayer

It is known that small hydrophilic molecules pass through a dense cellular monolayer by paracellular transport, that is, through dense cellular contacts. The Lucifer Yellow dye (LY, Lucifer yellow, L0259, Sigma or the like) belongs to this type of compound and can serve as a marker for assessing the integrity of a monolayer.

The approach is based on the joint exposure of cells with LY solution and the test object in the apical-basolateral direction. The amount of LY in the samples is determined fluorimetrically using a calibration relationship. Based on the obtained data, the permeability coefficient P_{app} (Apparent Permeability) is calculated.

The damaging effect of the test objects on the cell monolayer was evaluated by comparing the Papp calculated for it with the same value obtained for the control sample (cells exposed with LY only). P_{app} of the maximum non-cytotoxic concentration of the test object shall not exceed P_{app} control (LY). Based on the results of this part of the experiment, a working range of concentrations of test objects suitable for performing the main test was determined.



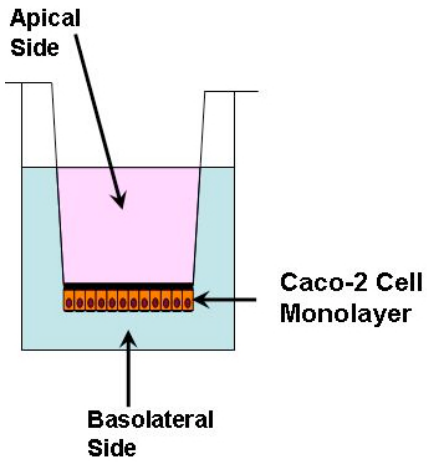
Cell permeability assessment (Caco2 model)

3. Evaluation of the cell permeability of the test object

To reproduce the conditions of the small intestine, experiments were carried out under pH gradient conditions. To create it, a buffer solution with a pH of 6.0-6.5 is used in the apical compartment, and a buffer solution with a pH of 7.2-7.4 is used in the basolateral compartment. A solution of the test object in a suitable solvent is added to the donor compartment; the system is incubated at temperature 37ºC; samples are taken from the acceptor compartment at certain intervals. The obtained samples were analyzed for the content of the added substances.

To confirm the adequacy of the test, experiments were performed with control substances (propranolol hydrochloride and sodium warfarin).

Assessment of permeability and transport of tested objects and control substances is performed in apical-basolateral (A-B) and basolateral-apical (B-A) directions.



Cell permeability assessment (Caco2 model)

To assess the cell permeability of the test compounds, the following calculations were made:

1. From the results of HPLC analysis, values of concentrations of the test compounds in μg/ml were obtained. For control substances, values were converted to μM.

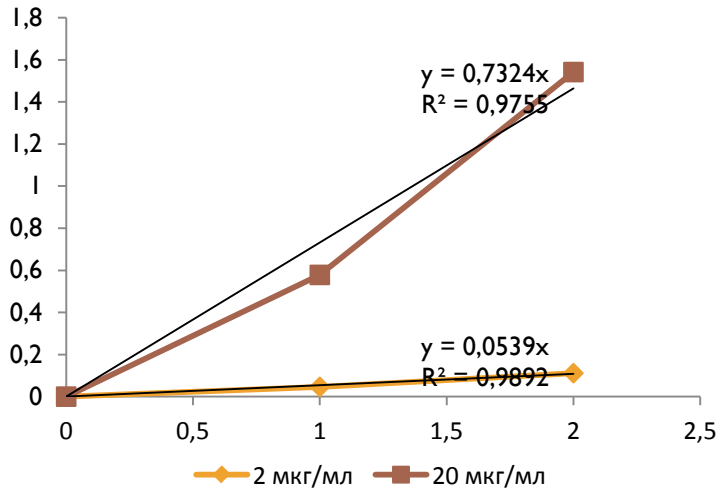
2. The concentrations of the test compounds were recalculated for each of the experimental points, taking into account the dilution of the obtained samples during the experiment. Dilution of the samples occurred because a portion of the compartment contents was taken at each time point and an equivalent volume of buffer solution was added.

3. Dependencies of the concentrations obtained from the results of calculating time after their introduction into the test system were built. Data were approximated using linear dependencies passing through the origin.

4. The obtained values of coefficients a of linear equations of the form y = a * x were used to calculate the permeability coefficient of the cellular monolayer (Rarp) according to the formula (3):

P_{app} [cm/sec] = Va/C0 x 1/S x dC/dt (3),

where Va - volume of acceptor part, 0.8 cm^3
for apical-basolateral or
0.2 cm^3 basolateral-apical direction;
C0 - concentration of the determined compound
in the donor part at T = 0 ("zero" point), μM;
S is membrane area, 0.33 cm^2;
dC/dt - angle of inclination of linear dependence
concentration of the detectable compound from
time in acceptance compartment
taking into account dilution, μM/s (coefficient a
corresponding linear equation of the form y = a * x)



Cell permeability assessment (Caco2 model)

Results of cell permeability assessment in Caco-2 model of propranolol hydrochloride in apical-basolaterial direction, P_{app} · 10-5, cm/sec

Concentration, μM	Experiment			Mean	SD
	1	2	3		
10	0,09	0,34	0,21	0,21	0,13
50	0,10	0,35	0,22	0,22	0,13
100	0,03	0,32	0,17	0,17	0,15
200	0,09	0,28	0,18	0,18	0,10

Results of cell permeability assessment in Caco-2 model of sodium propranolol in basolaterial-apical direction, P_{app} · 10-5, cm/sec

Concentration, μM	Experiment			Mean	SD
	1	2	3		
10	1,77	1,28	0,8	1,28	0,49
50	1,96	1,21	0,46	1,21	0,75
100	1,12	1,79	0,52	1,15	0,64
200	1,87	1,20	0,53	1,20	0,67

Cell permeability assessment (Caco2 model)

Results of cell permeability assessment in Caco-2 model of sodium warfarin in apical-basolaterial direction, P_{app} · 10-5, cm/sec

Concentration, μM	Experiment			Mean	SD
	1	2	3		
10	0,86	0,93	1,22	1,00	0,19
50	0,84	1,07	1,30	1,07	0,23
100	0,99	0,80	1,28	1,02	0,24
200	0,74	0,91	1,08	0,91	0,17

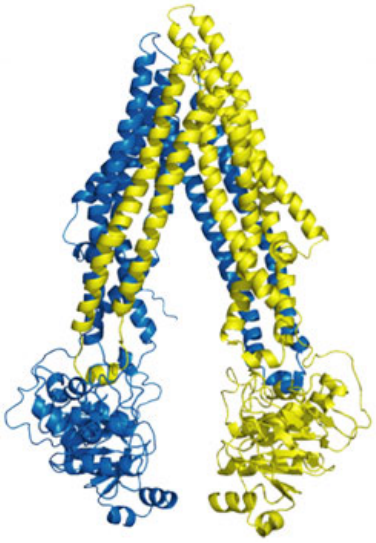
Results of cell permeability assessment in Caco-2 model of sodium warfarin in basolaterial-apical direction, P_{app} · 10-5, cm/sec

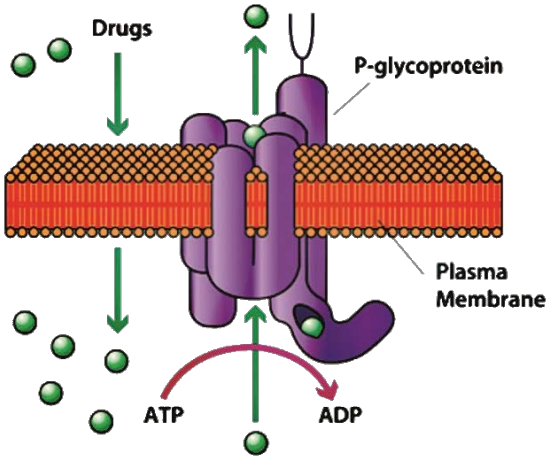
Concentration, μM	Experiment			Mean	SD
	1	2	3		
10	1,36	1,2	0,40	0,98	0,51
50	1,47	0,89	0,31	0,89	0,58
100	0,92	1,59	0,35	0,95	0,62
200	1,68	1,11	0,54	1,11	0,57

The results showed that the permeability of propranolol hydrochloride in the apical-basolaterial direction is less than sodium warfarin, which is consistent with the literature. Thus, the adequacy of the cell permeability test and its suitability for further evaluation of the test substances can be concluded.

Cell permeability assessment (Caco2 model)

If P_{app} in the basolateral-apical direction is greater than P_{app} in the apical-basolateral direction by 2 or more times, the compound can be a substrate for P-gp (i.e., transport can pass both inside the cell and outside).



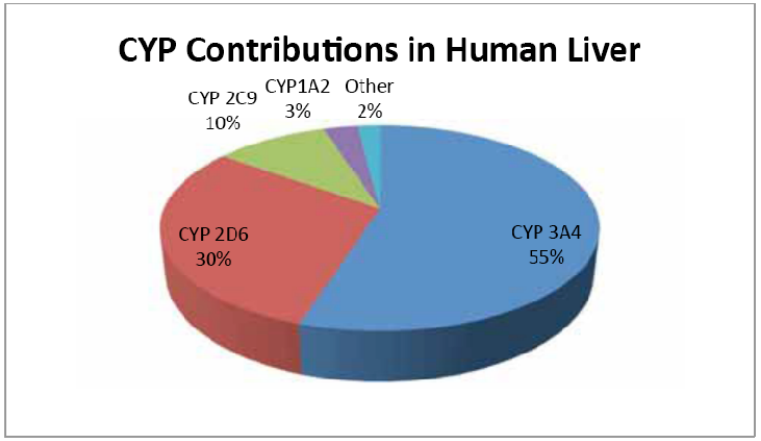


Minimum set of tests. What about the properties of the compound being studied?

- Binding to blood plasma proteins - suggested information on the presence of free/bound forms in the bloodstream
- Stability in liver microsomes - hypothesized information on metabolic rate
- Cell permeability assessment (Caco2 model) - hypothesized information on intestinal absorption and potential for reverse transport

Effects on cytochrome P450 - phenotyping

- with respect to 5-7 isoforms P-450: 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4.
-similar to the microsomal stability test; incubation with liver microsomes is carried out with and without the addition of selective inhibitors (ketoconazole (3A4), quinidine (2D6), α-naphthoflavone (1A2), miconazole (2C19, 2B6), sulfafenazole (2C9), 4-methylpyrazole (2E1)),
-remaining amount of the unchanged substance is determined, which isoform accelerates metabolism of the analysed compound.
-one concentration of human liver microsomes, one concentration of test compound is used during the test.
-- determining half-life ($T_{1/2}$), internal clearance (Clint), remaining amount of analyte (% of initial).



A diagram of the distribution of isoforms of P-450 in the human liver.
(After: Bowen J.P., Guner O.F. A Perspective on Quantum Mechanics Calculations in ADMET Predictions. Curr. Top. Med. Chem. 2013;13(11):1257-72.)

Effect on cytochrome P450 - inhibition evaluation

- the test is performed on 5-7 cytochrome isoforms P450 in the presence of their specific inhibitors and substrates;
several concentrations of the test substance are used;
After incubation, the content of specific metabolites corresponding to each substrate (HPLC/MS/MS) or using a fluorescent approach (Vivid ® CYP450 Screening Kits from Invitrogen, developed for each cytochrome isoform; based on the interaction of recombinant enzymes (CYP450 Baculosomes ®) and their specific fluorescent substrates (Vivid ® Substrates).
determining a% inhibition of formation of selected metabolites for each of the isoforms under the influence of the test compound; IC50 is the concentration at which enzyme activity is reduced by 50%.

Effect on cytochrome P450

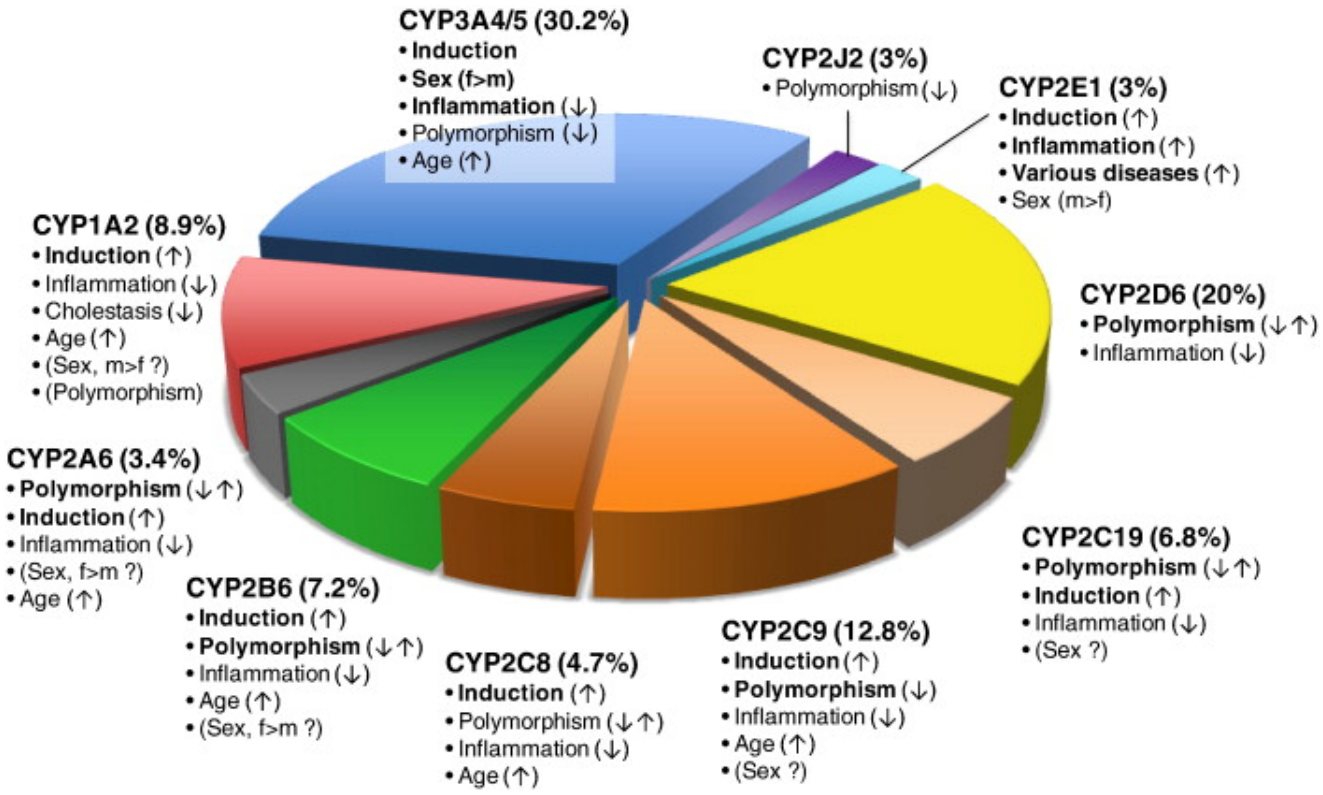


Diagram of clinically applied drugs (248 positions) metabolized by different cytochrome isoforms P450 and variability factors (after Zanger U., Schwab M. Cytochrome P450 Enzymes in Drug Metabolism: Regulation of Gene Expression, Enzyme Activities, and Impact of Genetic Variation. Pharmacoter.Res. 2014 138(1):103-41).

Conclusion

- ADME panel tests are important for studying the properties of potential drug candidates (original substances), generic and hybrid drugs, combinations of known substances;
- Reduce research using experimental animals;
- Their unification, comparison and synthesis of accumulated data is relevant.
- This minimum set of tests addresses compliance challenges as well as the practical challenges required for successful pharmaceutical development.

Thanks for attention!

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