THE STRUCTURE AND SCHEDULING OF PHARMACOKINETIC AND METABOLISM STUDIES

Pharmacokinetic and Metabolism Studies in the CHINOIN Co. Ltd.

Ph.D. Thesis

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PUBLICATIONS RELATED TO THE THESIS

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I. Koltai, E., Bánfi, D., **Szatmári, I.**, Hornyák-Hámori, M., Schawartz, J., Szüts, T.: Rapid synthesis of ^{14}C labelled 4(5)-amino-5(4)-imidazole-carboxamide. *Journal of Labelled Compounds and Radiopharmaceuticals*, **XVI.**, 777-780 **(1978)**

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LIST OF ABBREVIATIONS

ADME - Absorption, Distribution, Metabolism and Excretion
AMP - Amphetamine
AUC - Area Under the Curve
C_{max} - Peak concentration
CYP450 - Cytochrome P450
DES - Desmethylselegiline
DDT - 1,1,1-trichlor-2,2-bis-(4-chlorphenyl)-ethan
DIMEB - Dimethyl-β-cyclodextrin
DMF - Dimethyl formamide
D% - Percentage of the dose
EC - Electron-Capture
Eq. - Equivalent
FID - Flame Ionization Detector
GC - Gas Chromatography
GC-MS - Gas Chromatography equipped with Mass Spectrometry
GCP - Good Clinical Practices
GLP - Good Laboratory Practices
HLEI - Human leucocyte elastase inhibitor
HPLC - High-performance Liquid Chromatography
IV - Intravenous
LC-MS/MS - Liquid Chromatography equipped with tandem Mass Spectrometry
MET - Methamphetamine
MS - Mass Spectrometry
MSD - Mass Selective Detector
mv - Microvilli
N - Number of the elements
NMR - Nuclear Magnetic Resonance
NP - Nitrogen-Phosphorous
OGYI - Országos Gyógyszerészeti Intézet [National Institute of Pharmacy]
P_{app} - Apparent permeability coefficient
PEG - Polyethylene glycol
PEPI - Prolylendopeptidase inhibitor
PK - Pharmacokinetics
PO - Oral
SEL - Selegiline
SNO - Selegiline-*N*-oxide
TCDD - 2,3,7,8-tetrachlorodibenzo-p-dioxin
TEA - Triethylamine
tj - tight junction
TK - Toxicokinetics
TLC - Thin-layer Chromatography
TOT - Total
TRIS - 2-amino-2-hydroxymethyl-1,3-propandiol

1. INTRODUCTION

In the last two decades, there was a change in the paradigm of drug research, and this worldwide tendency has reached CHINOIN R&D at the beginning of nineties. According to the former concept some or maximum some tens of compounds were synthesized and examined for *in vivo* pharmacological activity and for toxic effects. That is functional pharmacology and toxicology were the bases of a selection for further development. Very frequently, we knew nothing about the mechanism of action, and we had the first (negative) results on the absorption and metabolism of the compounds during the first clinical trials, when the company already spent a lot of money for the development.
The principle of the new concept is mechanism-based or a molecular targeting research. Receptors, enzymes, ion-channels are the most frequent molecular targets, and the candidates are selected from some hundreds or thousands of compounds (compound-libraries, traditional synthesis and combinatorial chemistry provide more and more new chemical entities) based on their *in vitro* binding affinity.

It takes 12 to 15 years to develop a new drug and bring it to market. For each successful new drug, roughly 5,000 compounds are evaluated in biochemical assays *in vitro* and in animal studies. Of these, only five will be found to be suitable for human clinical trials, and only one of the five will gain approval by the Food & Drug Administration as safe to market[1]. According to the above, it can be understood that drug research is very expensive. The costs of the development of a drug are estimated to be about $500 million (or higher), but these also include all the costs of unsuccessful projects and compounds. More than one-half of the money invested in pharmaceutical R&D is spent on molecules that never reach the clinic, and practically, "only" $50 million are the expenses of one successful product. The costs rise precipitously in the later stages of the drug development process. Candidates that fail late in the development stage incur huge, unrecoverable expenses.
Roughly, 40 % of putative drug candidates – or new chemical entities – are rejected because of poor pharmacokinetics[1]. Similar data were published from the middle of nineties[2,3]. The rejected compounds do not make the grade in absorption, distribution, metabolism, or excretion. The 40 % rate is much higher than that of the compounds rejected due to the lack of human efficacy (30 %), toxic effects in animals (11 %), adverse effects in man (10 %), marketing reasons (5 %) or others (4 %).
After surveying the above statistical data, it becomes clear why the change in the paradigm of the drug research involved the change in the role of the pharmacokinetic studies in the whole research and development process, and fortunately enough, the new methods and techniques provide the background for this new concept.
When the new concept was accepted and applied in CHINOIN R&D – at the beginning of the nineties – a new approach in the pharmacokinetic and metabolism studies was also developed. This complex system of the ADME studies (absorption, distribution, metabolism and elimination) has been in use since then in the Pharmacokinetic and Metabolism Laboratory headed by me.
Before discussing the details of the pharmacokinetics and metabolism, I have to make two important notes, which can explain some specialties of this thesis. On the one hand, the results of the drug research and especially those before patenting the best candidates are highly confidential. Too much money is spent on a drug, and the competition is rather strong, too.

For these reasons no publication is generally allowed at this stage. This is why the results of our work related to the discovery phase were not published; only some obscure hints were presented as examples in symposia [XVIII, XVI]. On the other hand, drug research and development is not a job of lonely scientist. I have been the head of the Pharmacokinetic and Metabolism Laboratory since 1988, and my principal task is to manage the laboratory, that is to determine the system of the studies to be performed according to current scientific and regulatory requirements, and to ensure the conditions for the work. It is the task of my staff to perform certain studies, and they always do it rather high standards. Accordingly, the results of the studies performed after 1988, and I will present hereafter, are common results of a team, where my role is much rather the planning, management and evaluation of the studies than the experimental work.
In this paper, I will

- introduce the structure and scheduling the pharmacokinetic and metabolism studies, according to the new R&D approach
- show some examples how pharmacokinetic and metabolism studies help the selection of the drug candidates, the synthesis of new compounds having more favorable features
- present examples on the synthesis of radiolabelled tracer molecules
- introduce studies having the "classical roles" of the ADME studies, that is on the preclinical and clinical studies required for the registration of the drugs

2. PHARMACOKINETIC AND METABOLISM STUDIES

The traditional role of the pharmacokinetic and metabolism studies was to clarify the absorption, distribution, biotransformation and excretion of the compounds selected for the development, in animal experiments then in clinical trials. The studies were initiated in the so-called preclinical phase with the in vivo experiments in rats, mice and dogs.
According to the new concept, the pharmacokinetic examinations are started already in the discovery phase, and the traditional role is supplemented by a new one: to help the selection of the drug candidates. Thus, a general scheme of the drug discovery process can be demonstrated as it is depicted in Figure 1.

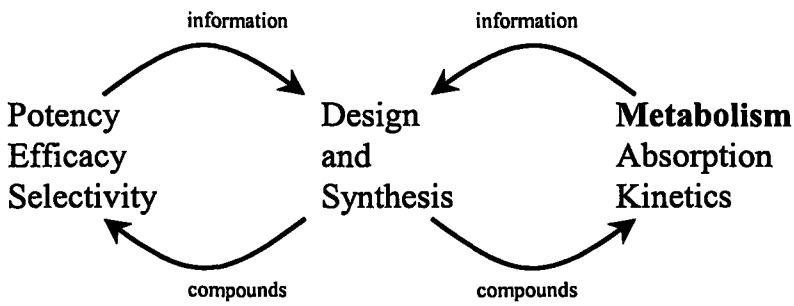


Figure 1. General scheme of the drug discovery process[4]

In our laboratory, both roles, the traditional one to provide safety results for the first human use or for the registration, and the new one to help the selection of drug candidates, were parts of our activities.

2.1. BIOANALYTICS

The basis of almost all pharmacokinetic and metabolism studies is the bioanalytical method.
In the sixties and seventies the use of test substances labelled with radioactive nuclides such as ^{3}H, ^{14}C, and sometimes ^{32}S or ^{125}I, and measurements based on the monitoring of the radioactivity were the most popular. Applying radiolabelled preparation, not only the unchanged molecule was measured, but also its metabolites containing the characteristic part where the radionuclide was built in. Due to this advantage, this analytical technique remained a good tool for the determination of mass balance and metabolic profile even today. However, this advantage is a kind of drawback of the technique. Measurement of radioactivity alone cannot distinguish the metabolites from the unchanged compound. This is the reason why measurement of radioactivity is combined with chromatographic techniques (HPLC, GC). Another disadvantage is that rather few compounds can be tested with this method, because the synthesis of the radiolabelled derivatives takes a lot of time and money.
Today, when the number of the hit drugs is huge, it is impossible to make radiolabelled preparation from all of them; thus, quicker, cheaper and more selective methods are necessary. HPLC or LC-MS/MS and — less frequently — GC or GC-MS provide a good tool for the quick and relatively cheap assays. These techniques can be used from the beginning to the end of the drug research and development process.

2.1.1. Synthesis of radiolabelled compounds

Radionuclides used most commonly in the pharmacokinetic and metabolism studies are ^{14}C and ^{3}H. These β-emitters have low energy radiation (0.018 MeV and 0.159 MeV for ^{3}H and ^{14}C, respectively[5]), thus they can be administered into the living organisms without a significant risk of any damage of the tissues and organs. They also have a relatively or definitely long half-life (12.2 and 5730 years for ^{3}H and ^{14}C, respectively[5]), which is appropriate for the analytical purpose.
The crucial point of the synthesis of a radiolabelled preparation is always the positioning of radionuclides. Sometimes not one single nuclide is built in a compound to have higher specific radioactivity (in this case two or more of the same nuclide are built in, e.g. two ^{3}H) [IX], or to have the possibility to distinguish the parts of the molecule (in this case two different nuclides are used, e.g. one ^{14}C and one ^{3}H) [VI]. It is important that the radionuclide must be in a characteristic part of the molecule, so the potential metabolic pathways must be take in consideration before synthesis. It needs a very close cooperation between the synthetic chemists and the pharmacokineticians.
I show two examples on the synthesis of radiolabelled preparation from our practice. One is a ^{14}C-labelled compound, [^{14}C]AICA phosphate [I], the other is a tritium labelled compound, [^{3}H]Ipriflavone [IX]. I will also show an example on the application of double-labelled preparations [VI].

2.1.1.1. Synthesis of ^{14}C-labelled AICA phosphate [I]

AICA [4(5)-Amino-5(4)-imidazolecarboxamide] phosphate was a promising liver protecting agent in the seventies and eighties, the development of which finally was stopped due to toxicological reasons. Nevertheless, in the seventies considerable efforts were made to clarify its pharmacokinetic and metabolism characteristics and ^{14}C-labelled preparation was synthesized. (This synthesis was made in 1978, when I worked as guest researcher at the Radiochemical Department of the Institute for Drug Research). To be in a stable position, the radionuclide was built into the imidazole ring. For this, a new synthesis route was developed using [^{14}C]potassium cyanide as starting material. The steps of the synthesis can be seen in Figure 2. More details can be found in a publication [I].

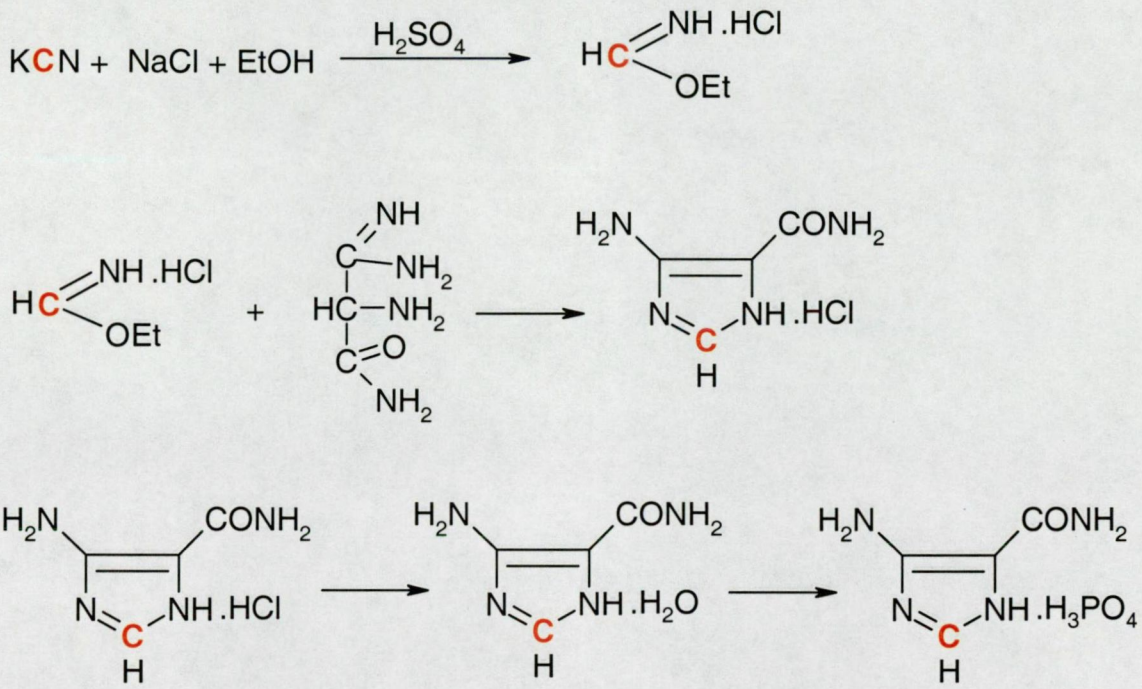


Figure 2. Synthesis route of [^{14}C]AICA phosphate [I]. ^{14}C-labelling is printed in red characters.

2.1.1.2. Synthesis of ^{3}H-labelled ipriflavone [IX]

Ipriflavone (7-*Iso*propoxyisoflavone) is an original antiosteoporotic agent of CHINOIN Co. Ltd., which was extensively examined also in our laboratory[6,7]. For metabolism studies and to examine the mechanism of action, tritium labelled preparation was synthesized. However, for these studies a preparation with high specific activity was necessary. Depending on the synthesis route, one or two radionuclides could be built into the molecule. The steps of the synthesis can be seen in Figure 3. The details are published in a paper in 1999 [IX].

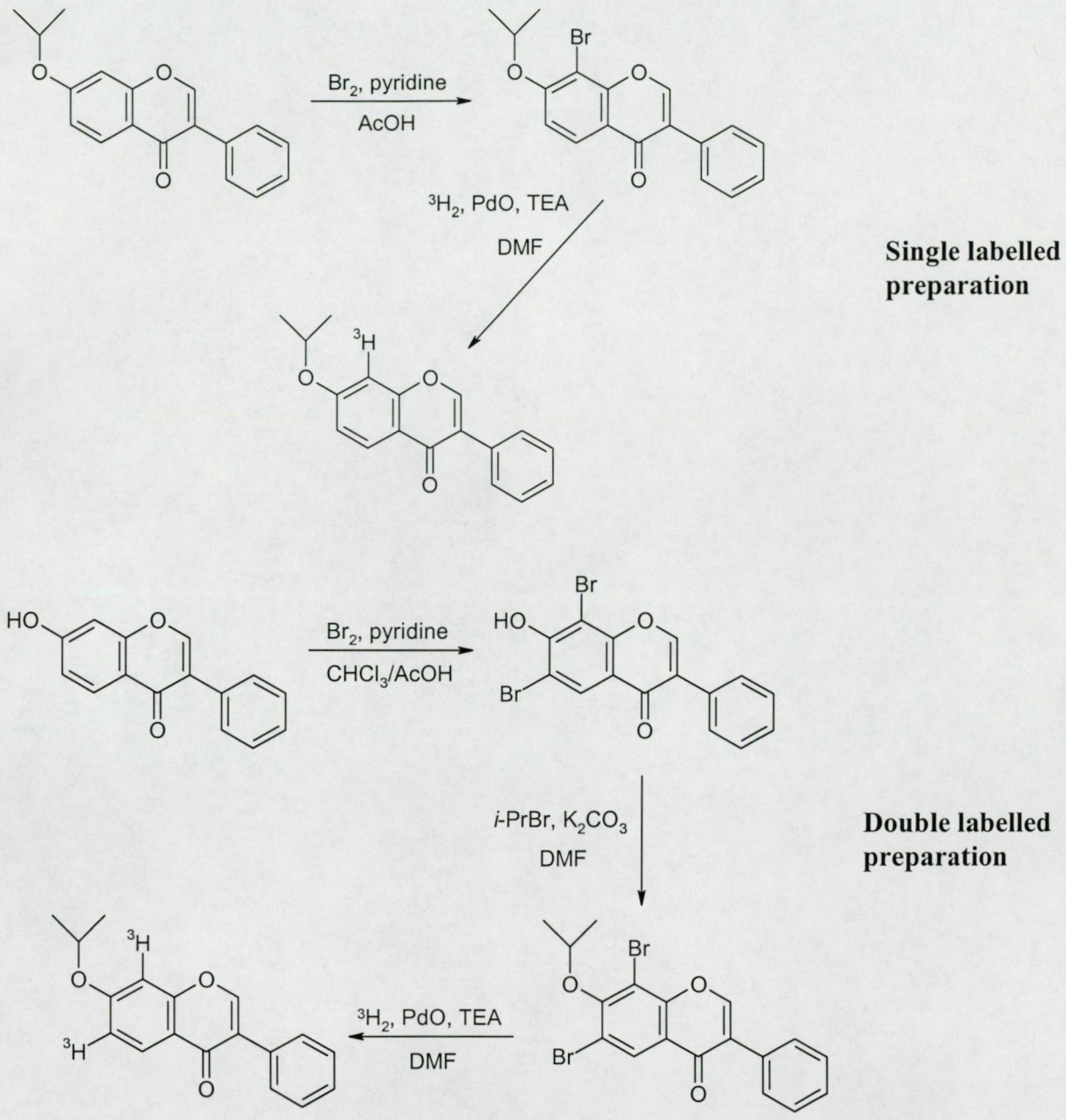


Figure 3. Synthesis routes of ^{3}H-labelled ipriflavone [IX]

2.1.1.3. Use of quasi-double-labelled preparation [VI]

Selegiline ((-)-deprenyl, JUMEX®) is one of the most successful products of CHINOIN Co. Ltd. It is a selective and irreversible inhibitor of monoamine oxidase type B, and it is widely used for the treatment of Parkinson's disease[8]. Chemically, selegiline has two characteristic parts. One is amphetamine-like group containing the phenyl ring, and the other is the propargyl part. Since N-depropargylation is a main metabolic pathway for selegiline [V, XI], it was important to know if unchanged compound and/or which metabolites can be found in the different tissues, and especially in the different regions of the brain. For this purpose, two

different radioactive preparations, selegiline-propynyl-^{14}C and selegiline-phenyl-^{3}H were synthesized (Figure 4). Creating a quasi-double-labelled preparation by mixing the two single-labelled materials, we could monitor the two characteristic parts of selegiline parallelly [VI, VII].

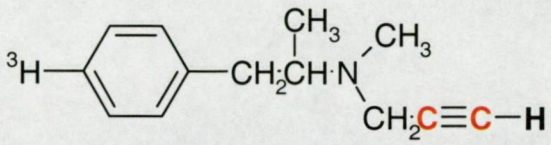


Figure 4. Positions of the ^{3}H and ^{14}C (red) atoms in the quasi-double-labelled selegiline preparation

2.1.2. Chromatographic bioanalytical methods [X, XIII]

Today chromatographic techniques are the most commonly used tools in the pharmacokinetic and metabolism studies. Unfortunately, the most modern technique, LC-MS/MS has been missing from our portfolio so far, but HPLC-s and GC-s equipped with various detectors (UV, fluorescence, diode-array, radioactivity detectors for HPLC-s, and FID, NP, EC, MSD for GC-s) can also be used efficiently.

The requirements for the bioanalytical methods are, however, different in the different phases of the research and development process. Less sensitive, but reproducible methods with relatively short run-time and with appropriate selectivity against the endogenous materials found in the biological matrices used are necessary for the assays in the discovery phase. For the determination of test materials in matrices from preclinical, and especially for toxicokinetic studies, as well as from clinical trials, the bioanalytical methods have to be fully validated and very sensitive. During the whole research and development process, often more methods are developed and validated for a compound, because getting on the development, first only the unchanged compound is measured, later on the pharmacologically active or toxic metabolites should be also determined. Different methods are maybe necessary for the determination of the test material in the various biological matrices (plasma, urine, tissues) and for different species (e.g. for rat, dog, and human).

The simultaneous HPLC determination of ipriflavone and its seven metabolites[7] [XIII] and the gas chromatographic method for the determination of deprenyl and its main metabolites[9] [X] can be good examples for the chromatographic methods developed in our laboratory.

2.2. Pharmacokinetic and metabolism studies in the discovery phase

Working according to the "molecular targeting research approach" — as it was already mentioned before — after the first biochemical screens, we know the mechanism of action and we know that the "hit" or "lead" compounds act on the target molecules *in vitro*. However, for the *in vivo* efficacy in human, the compound must be suited many requirements. It must reach the target (receptor or enzyme) unchanged, and stay there for long enough to interact with it.

The common pharmacokinetic and metabolism issues encountered during lead optimalization (to have the drug candidate) are concerned essentially with systemic exposure and the potential drug-drug interaction.
Since more hundreds or thousands of compounds have to be tested for their pharmacokinetics and metabolism, high or at least medium throughput *in vitro* techniques must be used. It is not only financial and practical requirement, but the aspects of animal protection are also mandatory.
The body is practically an infinite series of aqueous compartments bounded by lipid membranes, which membranes control the disposition of compounds. An optimal drug candidate has to be able to cross the membranes of the gastrointestinal tract (absorption), and different tissue membranes (distribution). The ability of the compound to cross these membranes is fundamental to reach the molecular target, as well as their presence at appropriate concentration. The optimal clearance of the compound resulting from metabolic and renal clearance is also essential. Too high clearance would cause short elimination half-life and short duration of action, while a too low clearance has the risk of drug accumulation.
To characterize the compounds synthesized for the lead optimalization, we examine the chemical stability, the absorption, the metabolic stability and the protein binding of candidate compounds.

2.2.1. Chemical stability

The aim of testing chemical stability is to know if the examined compound is stable enough in the experimental matrices and/or in the gastrointestinal tract, because the unchanged test compound must reach the molecular target. We cannot expect activity if the compound is destroyed before absorption. The primary matrices examined are as follows:

- pH 7.4 TRIS/HCl buffer used for the *in vitro* liver microsomal tests
- artificial gastric juice (0.2 g NaCl, 0.32 g pepsin and 2.5 g of 10 % HCl solution diluted with water up to 100 mL; pH = 2)
- artificial intestinal juice (1.0 g KH_2PO_4 and 1.5 g pancreatin in 100 mL of water adjusted to pH 7.5 with 0.1 M NaOH solution)

Stability of the compound is also tested in the vehicles used in the *in vivo* studies.

2.2.2. Absorption

From marketing point of view — based on the convenience of the application — the oral formulations are the most favorable, thus the development of an orally active drug is the primary aim of the pharmaceutical companies.
An optimal drug candidate first has to be absorbed from the gastrointestinal tract. A compound is considered to be absorbed if it reaches the systemic circulation unchanged. The systemic exposure can be poor if the transport through the intestinal membranes is limited or the parent compound is metabolized by "first pass" metabolism.
Several methods from *in vitro* to *in vivo* are used to assess the absorption of drug candidates. Recognizing that physicochemical properties are crucial in the absorption of a drug, the prediction based on some basic physicochemical parameters seems to be rather quick method. For example, Lipinski's Rule-of-Five is a very useful approach[10]. According to this paradigm,

poor intestinal absorption or permeability is expected if at least two parameters are out of range among of the following four ones:

- the molecular weight is higher than 500
- the number of hydrogen bond donors (sum of OH-s and NH-s) is higher than 5
- the number of hydrogen bond acceptors (sum of N-s and O-s) is higher than 10
- the calculated log P is higher than 5

However, even if this theory is a good approach, it marks the boundaries only.

The absorption of the compounds was formerly assessed mainly by their octanol-water partition coefficient (log D), or it was measured in different *in vivo* or *ex vivo* animal experiments, e.g. in isolated intestinal loops[11] or in intestinal perfusion techniques. Today one of the most widely applied method is the measurement of the permeability coefficient through a human colon carcinoma cell (CaCo-2) monolayer or its new subclone, CaCo-2/TC7. CaCo-2/TC7 cells have the favorable feature that — under strictly controlled conditions — they differentiate as small intestine endothelial cells, forming a monolayer with tight junctions among them. Figure 5. shows an electron microscopic picture of differentiated CaCo-2 cells[12], while Figure 6. presents a general setting of this absorption model.

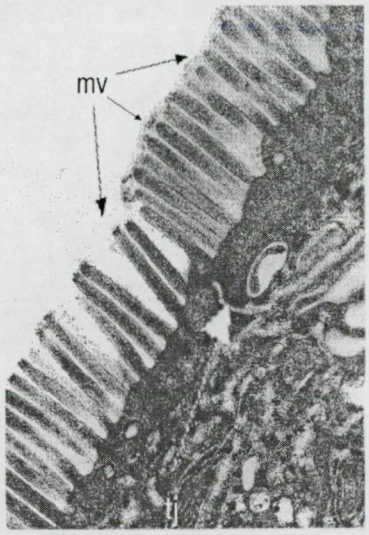


Figure 5. Electron micrograph of CaCo-2 cells[12]
(mv = microvilli, tj = tight junction)

The cells form a monolayer on a membrane; the test material is put into the apical medium, which represents the luminal side of the intestines. At appropriate time point(s), samples are taken from the basal medium to determine the quantity of the test material penetrated through the monolayer.

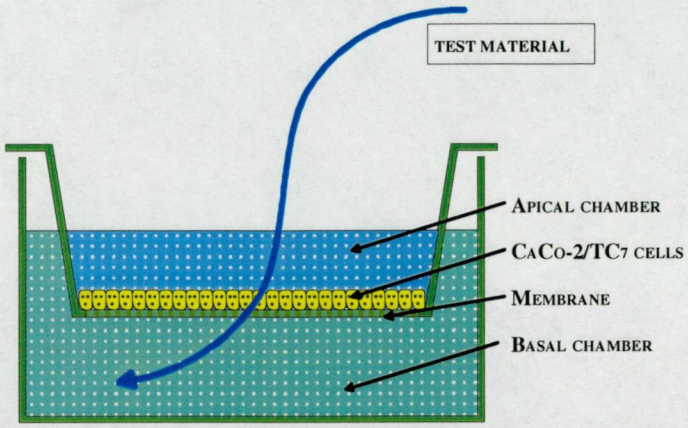


Figure 6. Experimental setting up of CaCo-2/TC7 *in vitro* absorption model

The monolayer formed by CaCo-2 cells has almost all the characteristics important for the absorption. It contains enzymes, which can metabolize the xenobiotics (exogenous compounds), active transporter molecules (e.g. P-glycoproteins), which can cause an efflux of the compounds penetrated the membranes, thus it can model all types of the absorption (para- and transcellular, active and passive transport, etc.). Test materials are applied in solubilised form, because the primary aim is to determine if the compound penetrates the membranes or not. For solubilization, we often use limited amount of organic solvents such as methanol or dimethylsulfoxide, since — according to the general trend — less and less water-soluble compounds arrive for study. Applying the test compounds in suspension, we can study the possible limiting role of the poor water solubility in the transport of the compounds through the membranes. When we put the test material into the basal medium, and take samples from the apical chamber (reverse arrangement), we can examine the efflux processes.
The penetration of the materials through CaCo-2 monolayer is characterized by the apparent permeability coefficient (P_{app}).

$$P_{app} = \frac{dQ}{dt} * \frac{1}{AC_0}$$

where A is the surface of the monolayer, C_0 is the initial concentration of the test material in the apical chamber and $\frac{dQ}{dt}$ is the amount of the test material passed to the basal chamber during the time period dt. This latter the following equation can be used:

$$\frac{dQ}{dt} = \frac{C_t * V}{t}$$

where C_t is the concentration of the test material in the basal chamber at time t. V is the volume of the basal medium.
Based on P_{app} values, the expected absorption of the test material can be assessed in human. P_{app} values of well-known drugs and chemicals, such as PEG4000, mannitol, terbutaline, amisulpride, furosemide, glutamine, alfuzosin, propranolol, metoprolol, verapamil, testosteron, antipyrine, mizolastine, and of some compounds developed in Sanofi-Synthelabo, such as SR140333, SR49059, SL25.1039, SR57746 and SR48692 were determined using the conditions detailed above. A sigmoid curve (Figure 7.) was prepared from these P_{app} values and the fraction of dose absorbed *in vivo* (F %) obtained from human investigations. This is

the basis of the categorization of the compounds tested. A compound is expected to be well absorbed from the human gastro-intestinal tract, if the $P_{app} > 2*10^{-6}$ cm/s, and no or poor absorption is expected, if $P_{app} < 1*10^{-7}$ cm/s.

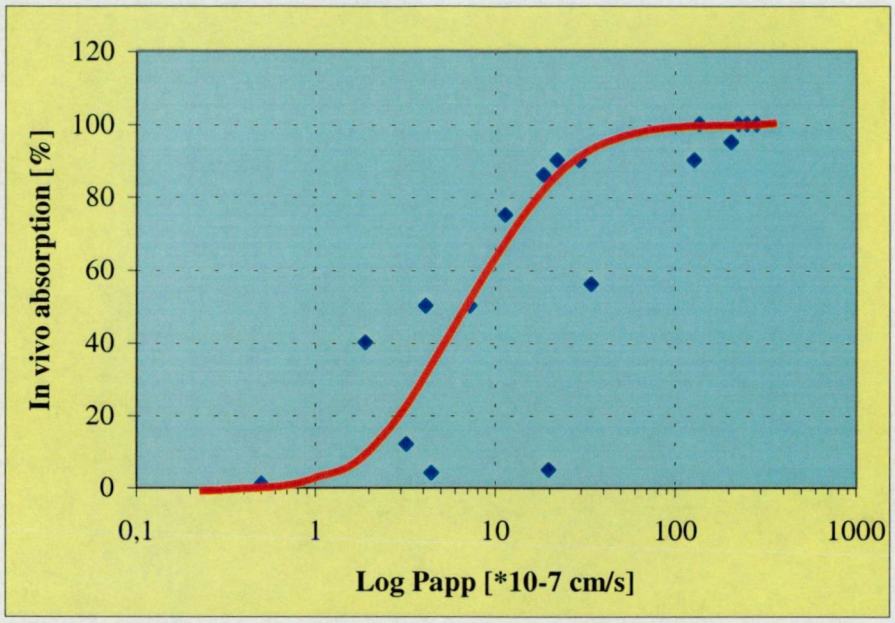


Figure 7. Correlation between the apparent permeability coefficient (P_{app}) and the fraction absorbed in vivo in human (F % values)

2.2.3. Metabolism

As regards *in vivo* activity, it is not enough if a compound is well absorbed from the gastrointestinal tract. It has to reach the target tissues unchanged, and it has to remain there unchanged for a certain time (it is not requested for the irreversible agonists or antagonists). About 90 % of the metabolic changes take place in the liver, but the kidneys, lungs, guts and blood (plasma) also can have significant metabolic activity.

Among the drug-metabolizing enzymes, cytochrome P450 (CYP450) is the most important. It was discovered in 1954 — while rat liver microsomes were examined — as "an appearing strong band at 450 nm"[13]. Since then we could learn that CYP450 is a superfamily of monooxygenase enzymes found across all living organisms and is postulated to have evolved from a common ancestral gene. Today several hundreds of CYP450 enzymes are known. The enzymes are divided into four families, and numerous subfamilies. They belong to the same gene family (gene family 1, 2, 3, 4, etc.) if they exhibit more than 40 % amino acid sequence homology, while those, which display more than 60 % (some authors give lower, 55 % percentage) sequence homology, belong to the same subfamily (e.g. subfamily 2A, 2B, 2C, 2D, etc.). However, among the more than 40 human CYP 450 enzymes known, only approximately ten have of importance in the metabolism of drugs. Table 1. summarizes the most important human CYP450 isoforms, as well as some of their characteristics, such as their abundance in the liver, proneness for polymorphism, inducibility.

Table 1. The known human cytochrom P450 isoenzymes and some of their characteristics[15,16,14]

CYP450 isoform	Abundance in liver	Ratio of the drugs metabolized		Poly-morphism	Inducibility	Inhibitors
		%	Examples			
1A1	0	9	Benzo[*a*]pyrene	- (?)	Isosafrole, Smoke, Charcoal-broiled food, Phenobarbital, Omeprazole, TCDD	1-ethynyl pyrene, Ciprofloxacin
1A2	9		Caffeine, Acetaminophen, Phenacetine, Lidocain	- (?)		Furafylline, α-naphthoflavone
1B1	<1	<1	Resveratrol, Chemical carcinogens	- (?)	Polyaromatic Hydrocarbons	Tranylcypromine, coumarine
2A6	4	1	Coumarine, Testosterone, Nicotine	+	Phenobarbital (?)	Tranylcypromine, ketoconazole,
2A7		-	-	-	-	-
2B6	<1	<1	Cyclophosphamide, Diazepam, Cocaine Ethylmorphine, DDT	-	Phenytoin, Clofibrate, Barbital, Dexamethasone, Phenobarbital	Quercetin, Chloramphenicol, Secobarbital, Orphenadrine
2B7						
2C8	16	15	Benzphetamine, Taxol, Diazepam, Diclofenac, Ibuprofen, Nifedipine, Warfarin, Mephenytoin	+	Dexamethasone, Phenobarbital, Rifampicine, Phenytoin, Secobarbital	Sulfaphenazole, Erythromycine
2C9						Sulfaphenazole, Amiodarone
2C19						Ticlopidine, Ketoconazole, Diazepam
2D6	2	30	Debrisoquine, Spartein, Metoprolol, Mexiletine	+	-	Quinidine, Desipramine, Fluoxetine, Pindolol, Simvastatin, Verapamil
2E1	6	1	Paracetamol, Halothane, Theophylline	- (?)	Fasting, Acetone, Ethanol, Isoniazid, Rifampicine	Disulfiram, Ciprofloxacin, Isoniazid
3A3	63	44	Cortisol, Cyclosporin A, Ketoconazole, Nifedipine	-	Carbazepine, Cortisol, Phenytoin, Dexamethasone, Ketoconazole, Phenobarbital, Rifampicine, Troleandomycin	Ketoconazole, Erythromycin, Cimetidine, Nifedipine, Naringenin, Troleandomycin, Amiodarone
3A4						
3A5			Erithromycin, Midazolam	+		
3A6			?	?		
3A7			?	+		

Isoenzymes having significant role in the human drug metabolism are **boldfaced**

Table 1. (Cont'd) The known human Cytochrom P450 isoenzymes and some of their characteristics[15,16]

CYP450 isoform	Abundance in liver	Ratio of the drugs metabolized		Poly-morphism	Inducibility	Inhibitors
4A9	<1	<1	Arachidonic acid, Lauric acid	-	Clofibrate, Ciprofibrate	-
4A11						
4B1						
4F2			Ebastine			
4F3						
5	?	?	Thromboxane synthase	?	?	?
7	?	?	Biosynthesis of steroid hormones such as progesterone, testosterone, corticosterone, estradiol	?	?	?
11A1						
11B1						
17A1						
19A1						
21A1						
27						

? No or conflicting data are available

The aim of the metabolic studies in the discovery phase is the assessment of the biological stability, that is to find a compound that can be expected to stay long enough in the organism, at the molecular target. The detailed studies of the metabolic processes are performed later, only with the selected compounds. For these reasons, the metabolic screens in the discovery phase are carried out using the most important biological matrices showing metabolizing activities, i.e. with the liver and plasma, and the experiments are aimed to answer the following questions:

- How labile is the compound to metabolic conversion?
- Are specific CYP isoforms such as CYP2D6 and CYP3A4 responsible for the biotransformation of the compound?
- Is the compound a potent inhibitor of specific CYP isoforms such as CYP2D6 and CYP3A4?
- Can glucuronidation be expected to be an important phase-2 metabolic process?

Plasma has a special position in the pharmacokinetic and metabolism processes due to at least two reasons. On one hand, blood (plasma) is the transporter of the drugs (except of the topical-acting, not-absorbing compounds), all drugs stay in plasma for a certain time, thus it is important to know if a compound remains unchanged in the plasma inside the body. On the other hand, pharmacokinetic and pharmacodynamic parameters are generally determined by the plasma levels of the parent compounds and their metabolites (except of the irreversible inhibitors of receptors or enzymes). For this reason, serial blood samples are taken from the species examined after oral and intravenous administration, and the bioavailability of the drugs is assessed by comparing the plasma concentrations. It is important to know if a compound is stable in the plasma collected.
Several techniques are used in the different research sites for the assessment of the hepatic metabolism. Precision-cut liver slices are almost complete systems, but their handling is somewhat difficult. Isolated liver cells can be easily used, and they are very popular in the drug discovery phase. They also can be considered as complete systems, having not only the microsomal but also cytosolic enzymes plus membranes and proteins participating in hepatic uptake. However, their preparation and maintenance are rather tedious. If we always prepare fresh primary cultures, the comparison of the results can cause problems, contrary, if we prepare a culture to use it for long period, the cells can lose their metabolic activities very soon.
Liver microsomal preparations contain the majority of the metabolizing enzymes participating in the biotransformation of xenobiotics. This preparation can be prepared easily; its metabolic activity can be preserved by freezing. The diversity of the enzymatic activity among individuals (mainly in the case of human beings) can be diminished by preparation of pools.
Owning to the ease of use, the primary assessment of the hepatic metabolism is often performed using liver microsomal preparations.

2.2.3.1. Metabolic stability

There are profound interspecies differences in CYP450 isoforms that make it almost impossible to extrapolate the findings from experimental animals to humans. For these reasons, we perform the *in vitro* metabolism studies in human preparation, as well as in preparations of animal species used in either the toxicological or pharmacological studies (rat,

mouse, dog, rabbit, hamster, guinea pig, gerbil). The metabolic stability of a drug in human and in animal species is compared, and we can get the first impression that the animal species to be used for the toxicological and pharmacological studies are adequate or not in this aspect. An example from our practice well demonstrates the above case. In the human leucocyte elastase (HLE) inhibitor project, CHINOIN researchers tried to find orally active inhibitors of HLE, supposing that these compounds are active in several illness including obstructive pulmonary diseases. The pharmacological test used also by the competitors was the so-called elastase-induced emphysema model in hamster[17,18]. It was surprising that a series of newly synthesized compounds were very active in the *in vitro* biochemical tests, but not in the hamster pharmacological model. The lack of the pharmacological activity even after intravenous administration excluded the first idea that the compounds had poor absorption. Later, the good absorption characteristics were confirmed by *in vitro* absorption studies. The *in vitro* metabolism studies in plasma (Figure 8.) and in liver microsomal preparations (Figure 9.) gave the explanation. The metabolism of drug candidates, and especially that of SSR69071 showed marked species differences. While SSR69071 was rather stable in human, dog and mouse plasma, as well as in human, dog, rat and mouse liver microsomal preparations, it was metabolically unstable in hamster and rat plasma, as well as in hamster liver microsomal preparation. Based on these results, change of the pharmacological species (and the pharmacological model) was suggested. Using rat and mouse models, a series of drug candidates having excellent *in vitro* biochemical activity, was also found to be active in the *in vivo* pharmacological tests. Finally, SSR69071 was selected for development.

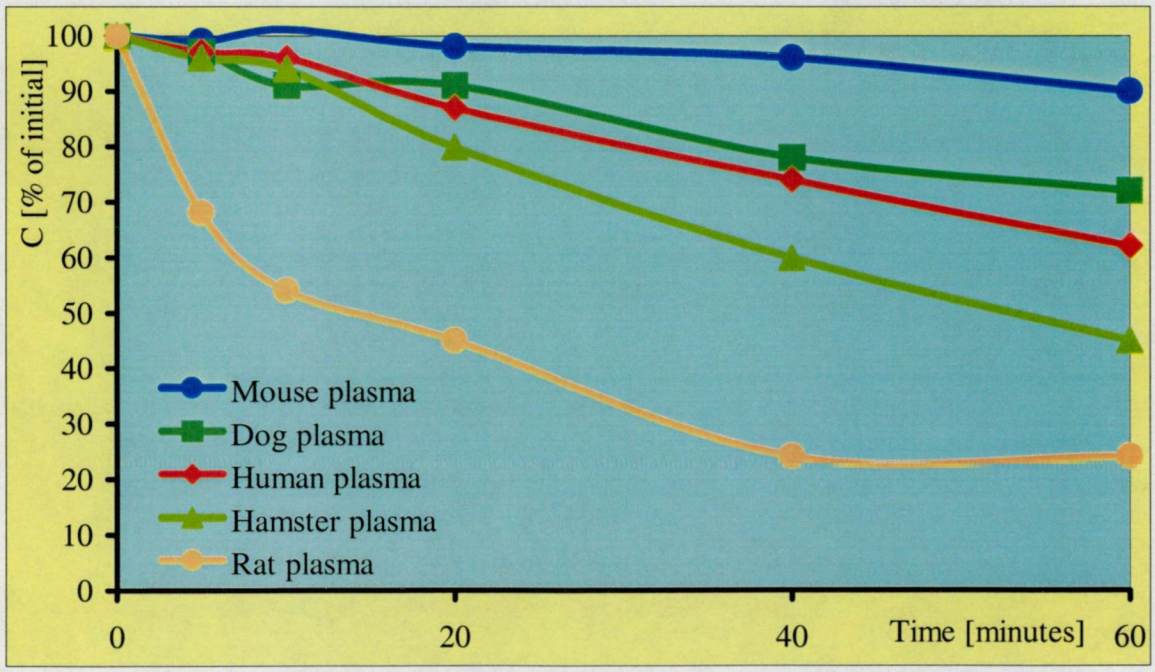


Figure 8. Metabolic stability of an HLE inhibitor, SSR69071 in plasma obtained from human and animal species

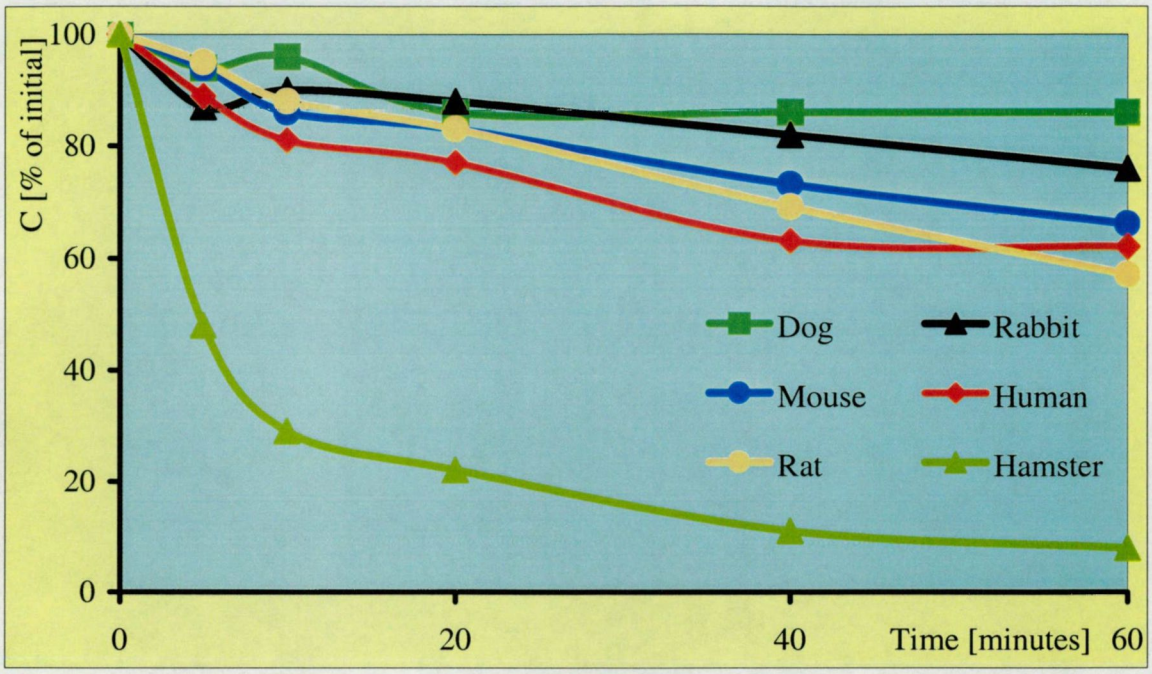
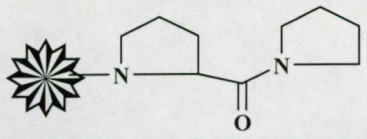
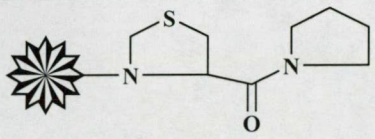
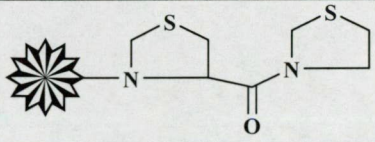
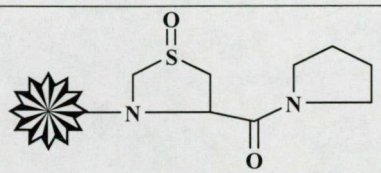
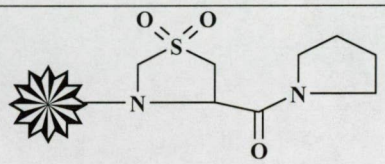


Figure 9. Metabolic stability of an HLE inhibitor, SSR69071 in liver microsomal preparation obtained from human and animal species

2.2.3.1.1. Chemical structure - metabolic stability relationship [XVIII]

It is a key question in the discovery phase of the drug research and development, how can we modify the chemical structure of a so-called hit compound for getting more potent, or more selective, or better absorbed, or metabolically more stable derivatives.
In the CHINOIN Co. Ltd., the aim of a project called prolylendopeptidase (PEP) inhibitors was to find potent, selective and orally active inhibitors of PEP enzyme. Following the synthesis of dozens of compounds, a lead compound having a bulky group (2-ethyl-phtalimide) and a prolyl-pyrrolidine part was selected for further optimalization. One of the possible modifications was introducing a sulfur atom into the prolyl and/or the pyrrolidine group. Our task was to test if the new derivatives having sulfur atoms were metabolically stable enough. Table 2. shows the metabolic stability results obtained in human and mouse liver microsomal preparation [XVIII]. The results proved that the metabolic stability is decreased by the number of the sulfur atoms introduced. However, the stability returned, if oxidized forms of sulfur atom were built into the compounds (formation of sulfoxide and sulfone is certainly a pathway of their metabolism).

Table 2. Chemical structure — metabolic stability relationship in human and mouse liver microsomal fraction [XVIII]

Chemical structure of the PEP inhibitors	Half-life of the metabolic degradation [minutes]	
	HUMAN	MOUSE
	365	55
	136	34
	87	15
	330	231
	210	96

2.2.4. Protein binding

Drugs bind to several biopolymers in the organisms. They bind to receptors and enzymes, which is the basis of their effect. They can also bind to the serum proteins, such as albumins, globulins, α-acyl-glycoprotein. The binding of a drug to serum proteins has a role in the transport of the drug, and in the formation of a depo. Thus, protein binding is a part of the distribution process. Furthermore, P-glycoproteins have a significant role in the membrane-transport of the drugs, and especially in the excretion processes and the transport through the blood-brain barrier.

There are several techniques for the examination of the binding of a drug to serum proteins. Equilibrium dialysis, ultracentrifugation, simple and dynamic ultrafiltration methods, gel chromatography and capillary electrophoresis are the most common methods.

At discovery phase, protein binding tests are performed only in special cases, when — based on the *in vivo* pharmacological or toxicological observations — protein binding seems to be a significant factor in the efficacy of the drug tested. In our laboratory, ultrafiltration method was selected for the screens, due to its simplicity and its low time-consumption.

2.2.5 In vivo bioavailability

In vivo bioavailability, in fact, is a kind of confirmation of the results of the *in vitro* absorption and *in vitro* metabolism studies. Using a minimum number of sampling times (4-5 sampling times) and a simple, partially validated analytical method for the assays, the absolute (e.g. oral versus intravenous) or relative (e.g. oral versus intraperitoneal) bioavailability is assessed.

2.3 PRECLINICAL PHARMACOKINETICS AND METABOLISM

Pharmacokinetic and metabolism studies performed in the preclinical phase are necessary for having approvals to initiate the first human trials. In fact, these studies can be called as classical ADME studies. These belong to the so-called safety studies, which are performed in animal species. In order to demonstrate preclinical pharmacokinetic and metabolism studies, results obtained from experiments with an antitussive and antiasthmatic preparation are briefly described hereafter. Other examples will be shown only in certain cases.

Antitussives are a traditional therapeutical field of CHINOIN Co. Ltd. Prenoxdiazine (LIBEXIN®) has been a widely used drug for years. In the eighties, CHINOIN started to develop new antitussive, antiasthmatic and mucoregulatory derivatives by combining the anti-asthmatic theophylline and antitussive prenoxdiazine. Numerous very effective members of the series were synthesized and characterized[19,20,21], out of them CHINOIN-170 (1,3-dimethyl-7-[(5-methyl-1,2,4-oxadiazole-3-yl)methyl]-3,7-dihydro-1*H*-purine-2,6-dione) and CH-13584 (3-methyl-7-[(5-methyl-1,2,4-oxadiazole-3-yl)methyl]-3,7-dihydro-1*H*-purine-2,6-dione)[20,22,23] were the most successful.

CHINOIN-170 was studied in details for animal pharmacokinetics[24], for animal metabolism[25] and for human pharmacokinetics[26]. Nevertheless, the project was stopped before registering the drug.

CH-13584 was found to be a major metabolite of CHINOIN-170, and it was found to be much more effective[19,20,25] than CHINOIN-170. Its non-clinical pharmacokinetic and metabolism characteristics were also studied in details [XII, XIV, XV].

The chemical structure of the two compounds can be seen in Figure 10.

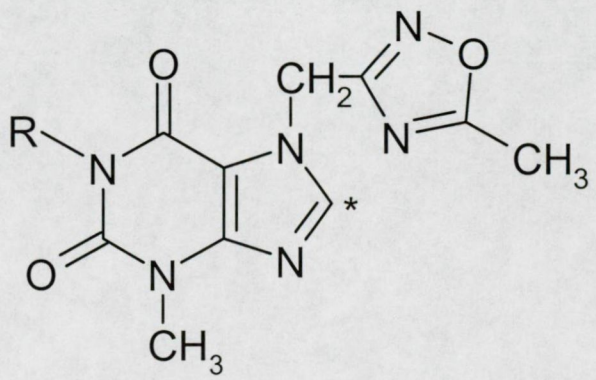


Figure 10. Chemical structure of CHINOIN-170 (R = CH_3) and CH-13584 (R = H) (* indicates the position of ^{14}C labeling when radiolabelled preparation was used)

2.3.1. Toxicokinetics

During the toxicological studies, the toxicological species are treated with rather high doses of the test material in order to clearly determine the highest dose having no toxicological effect, as well as the type of the toxic effects caused by the drug applied in higher doses than the "no-effect dose". There are two main reasons why a drug does not show any toxic effect even at high doses. First, it can be really a non-toxic drug, or there is no exposition of the animal by the drug studied, e.g. due to the lack of the absorption. To demonstrate the exposure, and to determine if the absorption of the drug studied is proportional with the administered dose, toxicokinetic studies, as a support of toxicological and safety pharmacological studies, are performed. Carrying out of these trials is obligatory for all of the toxicological studies performed in compliance with the principles of "Good Laboratory Practices" (GLP)[27,28]. The toxicokinetic studies have also to be performed in compliance with the principles of GLP.

2.3.1.1. Toxicokinetics of CH-13584 [XII]

The oral toxicity of CH-13584 was found to be very low, thus, the toxicokinetic studies had to answer if this low toxicity was due to the lack of the absorption.
Using a validated HPLC-UV method, concentration of the unchanged drug was measured in plasma samples obtained from male Wistar rats or from female beagle dogs. Both rats and dogs were treated orally with CH-13584 at single doses of 10, 100, 1000 and 2000 mg/kg. The results are summarized in Figure 11. and Figure 12.

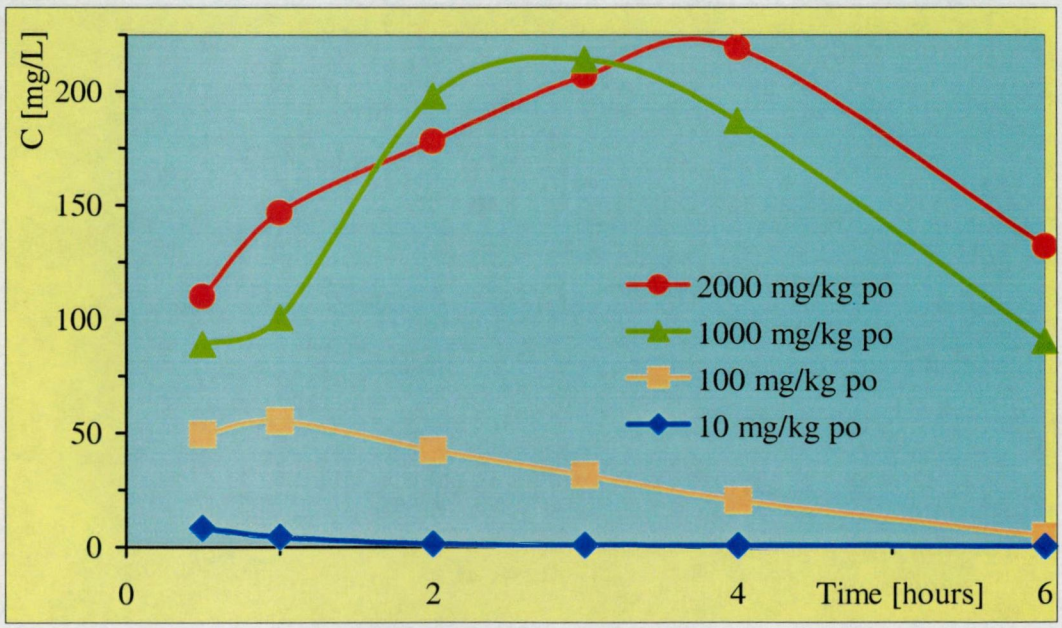


Figure 11. Plasma concentration versus time curve of unchanged drug in rat plasma after single oral administration of CH-13584

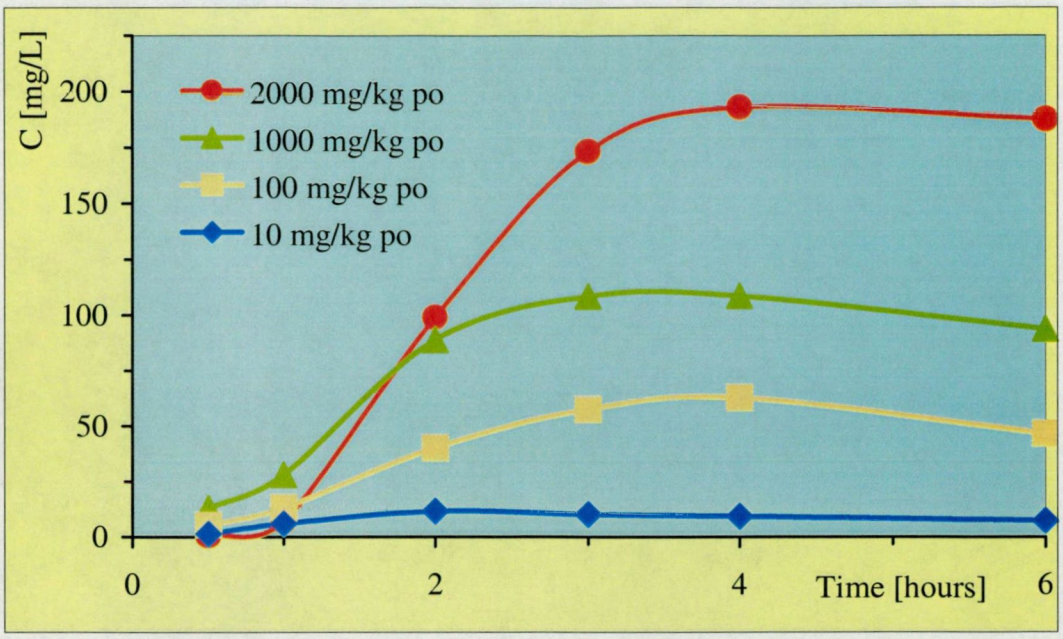


Figure 12. Plasma concentration versus time curve of unchanged drug in dog plasma after single oral administration of CH-13584

Based on the maximal plasma concentration values (C_{max}, Table 3.), it can be stated that absorption of CH-13584 is not linear within the dose range of 10 to 2000 mg/kg, either in rats or in dogs. However, the extent of the absorbed test material is increased up to 1000 mg/kg in rats, and up to 2000 mg/kg in dogs, but the linearity factors are far from 1.0. The results, however, prove that the reason of the low toxicity of CH-13584 is not the lack of the absorption.

Table 3. C_{max} values of CH-13584 in rats and dogs, and linearity factors of the absorption

Dose [mg/kg]	RATS				DOGS			
	C_{max} [mg/L]	Linearity*			C_{max} [mg/L]	Linearity*		
		/10	/100	/1000		/10	/100	/1000
10	11.5	-	-	-	11.8	-	-	-
100	56.3	0.49	-	-	58.6	0.49	-	-
1000	201	0.17	0.36	-	109	0.09	0.19	-
2000	204	0.09	0.18	0.51	202	0.09	0.17	0.93

* Linearity was calculated by the equation of $\frac{Dose(1) * C_{max}(2)}{Dose(2) * C_{max}(1)}$

2.3.2. Absorption, distribution and excretion

The non-clinical pharmacokinetic and metabolism studies have to be performed in one rodent and in one non-rodent animal species. Rat and the dog are used as typical species, but others (e.g. mice and monkeys) may be also used, depending on the drug candidate and the therapeutic field. There is no strict regulation on the methods and scheduling of the studies, but it is clear that data should be provided on the absorption, distribution and elimination of the unchanged test material and its metabolites. In most cases, the first studies are performed using radiolabelled test materials (see paragraph 2.1.1). These data are used for the first estimation of the fate of the drug in the human body, as well as for planning human trials.

2.3.2.1. Absorption

The assessment of the extent and the rate of the absorption of a drug is generally based on the plasma concentration of the total radioactivity and/or the unchanged material. Comparing the results obtained after oral and intravenous administration, we can calculate the bioavailability of the drug. However, absorption is only one of the factors affecting bioavailability. A compound absorbed completely can have low bioavailability, if it undergoes intensive "first pass" metabolism.
We can also have data on the extent of the absorption by calculating the area under the blood (plasma) curve (AUC); if we compare the elimination routes after oral and intravenous administration; or if we compare the parent compound content of feces and bile.
Finally, there are several methods for the direct measurement of the absorption.

2.3.2.1.1. Blood level monitoring of [^{14}C]CH-13584 in rats [XIV]

By monitoring the blood radioactivity levels in rats after different oral doses of [^{14}C]CH-13584 (2, 10 and 50 mg/kg), a nearly linear absorption can be found (Figure 13.). In Figure 14., it can be seen that no significant gender differences could be found, but the absorption is somewhat slower in females.
Comparing the area under the curve (AUC) values obtained after oral and intravenous administration approximately 90 % and 70 % of absorption can be seen in male and female rats, respectively.

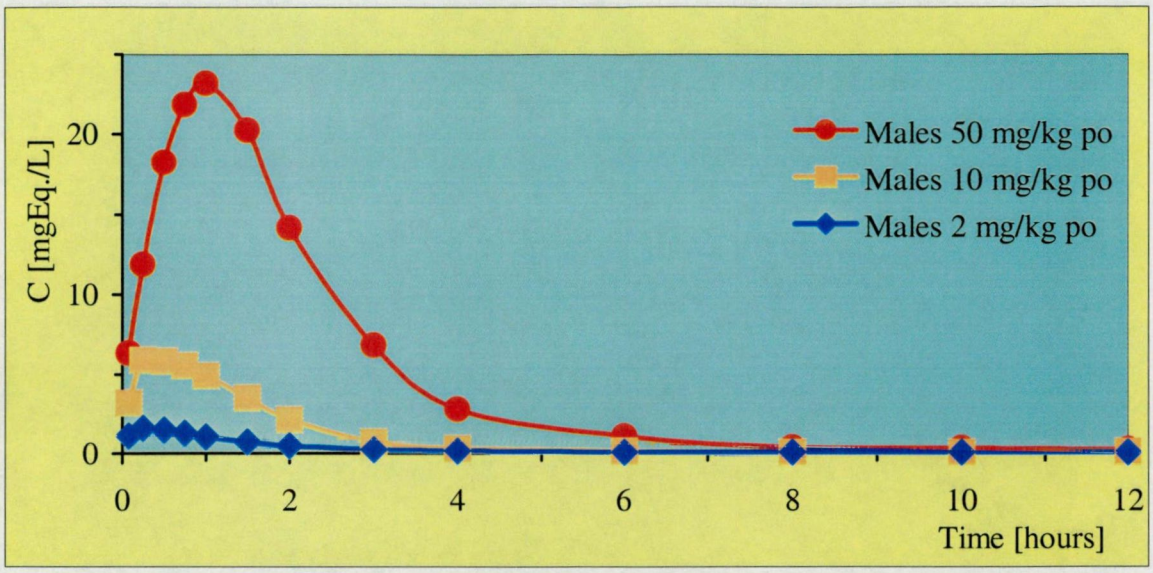


Figure 13. Blood radioactivity levels in male rats after single oral administration of [^{14}C]CH-13584 at the doses of 2, 10 and 50 mg/kg

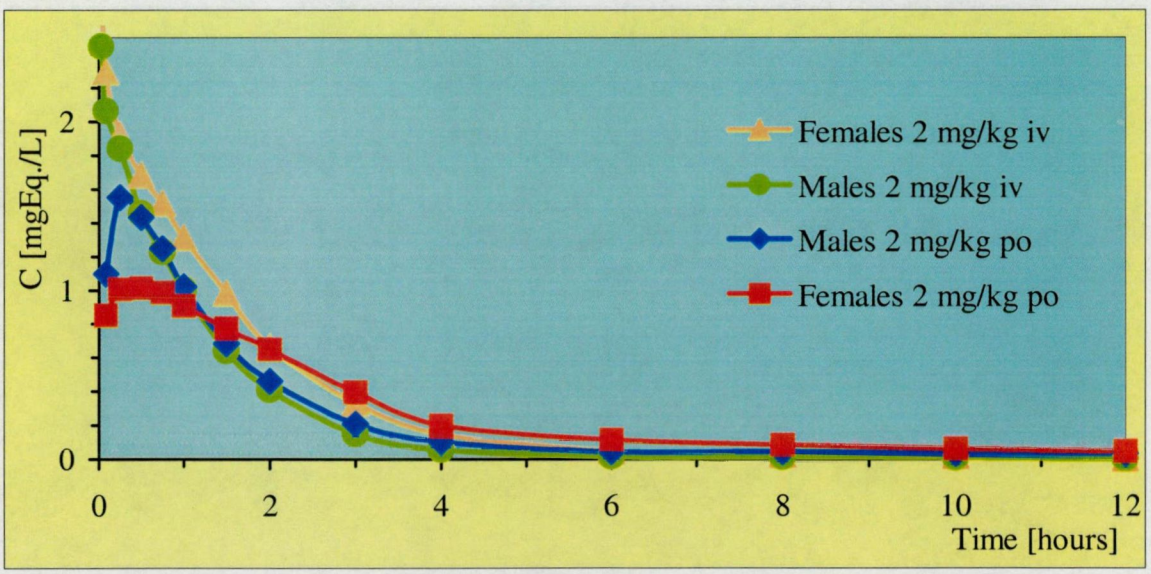


Figure 14. Blood radioactivity levels in male and female rats after single oral or intravenous administration of [^{14}C]CH-13584 at the dose of 2 mg/kg

2.3.2.1.2. Direct measurement of absorption in rats [II]

Direct measurement of the extent of the absorption is not a usual study; however, sometimes it can give very useful information. When drotaverine acephyllinate (also called as Depogen®); a new salt form of the well-known No-Spa® was studied in rats, the so-called "in vivo loop" technique was used to determine if the compound is absorbed from the small intestines or not. The results (Table 4.) proved that drotaverine is quickly absorbed from both the duodenal and

ileal parts of the small intestines, if administered as theophylline-7-acetic acid salt, directly into the experimental segments [II].

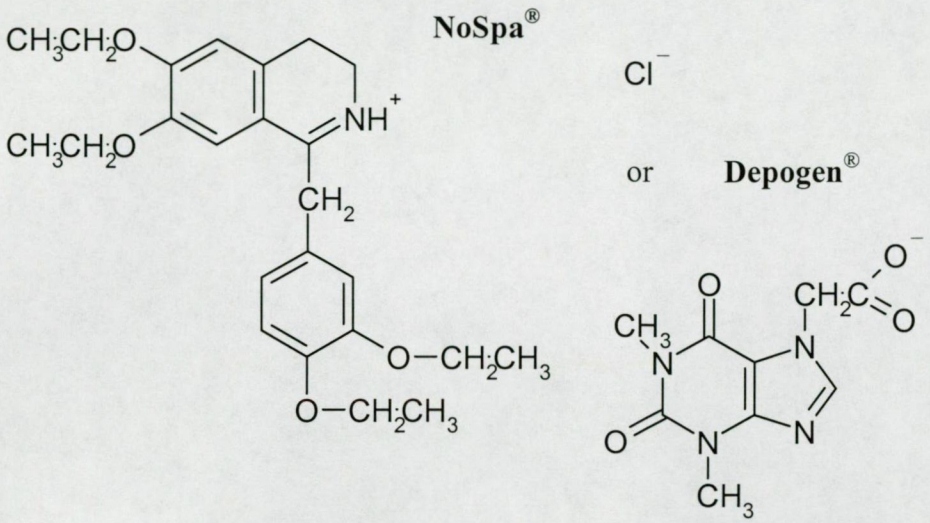


Figure 15. Chemical structure of NoSpa® and Depogen®

Table 4. Absorption of [^{14}C]drotaverine acephyllinate (80 μg/sac) from duodenal and ileal sacs (% of the dose administered, mean ± SD) [II]

Experimental segment	10 minutes	12.5 minutes	20 minutes	30 minutes
Duodenum	76.3 ± 5.9 N = 7	77.6 ± 8.0 N = 6	82.3 ± 10.3 N = 6	88.5 ± 2.9 N = 7
Ileum	68.0 ± 3.3 N = 5	82.1 ± 6.2 N = 6	84.2 ± 4.6 N = 7	92.4 ± 3.4 N = 7

2.3.2.2. Distribution

Understanding the organ and tissue distribution of a drug administered is very important due to several reasons. First, it is important if the pharmacologically active material reaches the target organs or tissues. Then, as a support for toxicological studies, it can be tested which organs or tissues are exposed by the test material or its metabolites. Finally, the rate of the distribution is a significant factor concerning the course of the plasma levels of the test material.

Two main methods are used to determine the tissue distribution of a drug.

Whole body autoradiography is an attractive method. Autoradiograms (visualized radioactivity maps) show the localization of the radioactive material representing the studied drug and its metabolites. These autoradiograms are made by exposing X-ray film or other

radiation-sensitive layer, or by direct measurement of radioactivity visualized by computers (Berthold Digital Autoradiograph). However, it is only a qualitative or semi-quantitative method, despite of the efforts devoted to make it quantitative.
The second technique is based on the direct radioactivity measurement of the organs and tissues. Certainly, special methods, such as digestion (solubilization), decolorization, and combustion are necessary to make the organs and tissues measurable by the liquid scintillation counter. This method, however, is quantitative.

2.3.2.2.1. Whole body autoradiography of [^{14}C]CH-13584 in rats [XV]

Distribution of radioactivity was studied in male and female, as well as in pregnant female Wistar rats. Ten mg/kg single oral dose was applied.
The results suggested rapid and intense penetration of radioactivity into the tissues and organs. The highest levels were detected in the gastrointestinal tract, hypophysis and thyroid gland. The initial rapid penetration was followed by a rapid elimination. High radioactivity levels could be detected only in the liver, kidneys and urinary bladder, in the so-called excretory organs. Twenty-four hours after the administration, the elimination of radioactivity was practically complete. No radioactivity could be found in the brain, however, radioactivity penetrated the placenta.
Among the autoradiograms prepared in this study, only three are presented. In order to help recognizing the organs, the photo of the same sections can be also seen below the whole-body autoradiograms.
Figure 16. shows the autoradiogram of a male rat obtained 0.5 hour after a single oral treatment with [^{14}C]CH-13584. Figure 17. shows the autoradiogram of a female rat obtained 3 hours after the treatment, while Figure 18. shows the autoradiogram of a pregnant dam obtained 1 hour after the treatment.

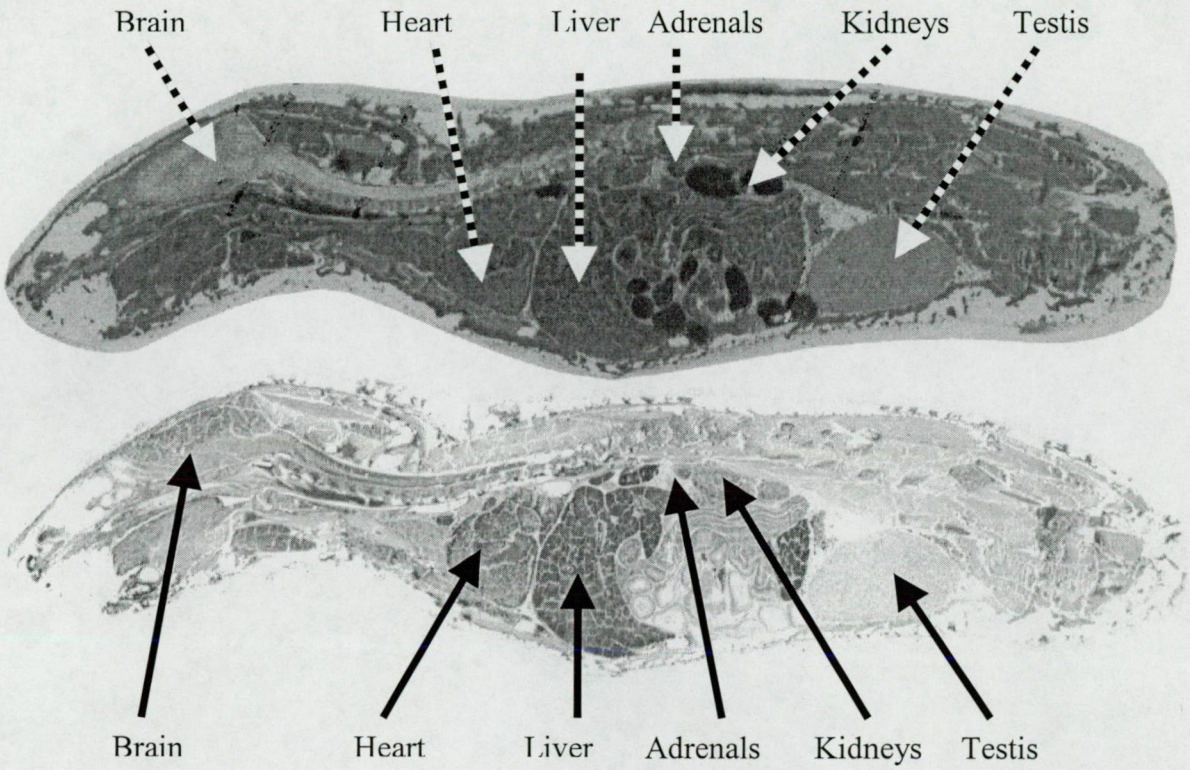


Figure 16. Whole body autoradiogram and the native photograph of a male rat obtained 0.5 hour after single oral treatment with [^{14}C]CH-13584

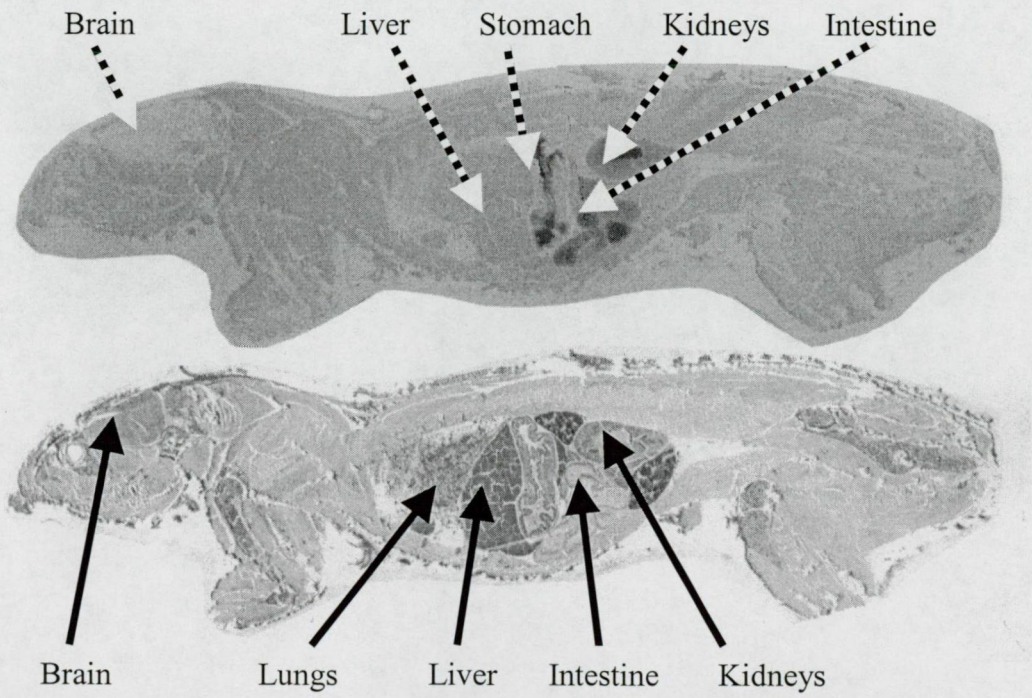


Figure 17. Whole body autoradiogram and the native photograph of a female rat obtained 3 hours after single oral treatment with [^{14}C]CH-13584

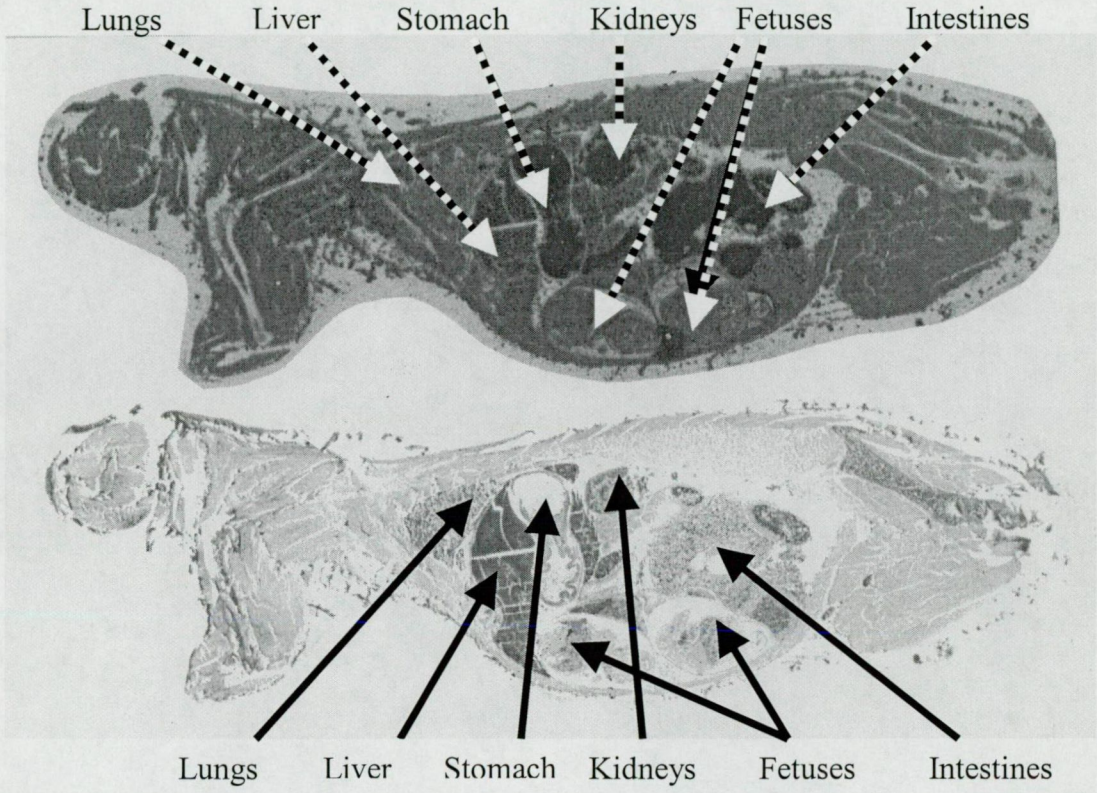


Figure 18. Whole body autoradiogram and the native photograph of a pregnant dam obtained 1 hour after single oral treatment with [^{14}C]CH-13584

2.3.2.2.2. Quantitative tissue distribution of [^{14}C]CH-13584 in rats [XIV]

Male and female Wistar rats were treated orally with 10 mg/kg dose of [^{14}C]CH-13584. Half an hour, 3, 6 and 24 hours after the treatment the animals were sacrificed, and the following organs and tissues were removed and measured for radioactivity: brain, heart, lungs, spleen, liver, kidneys, adrenals, thymus, stomach, small and large intestines, sexual organs (testis or ovaries), muscle and fat. Before measurement of radioactivity, the tissues were homogenized in saline solution using a blade homogenizer, then the aliquots were solubilised in Soluene 350®.

The results (Table 5.) are in good accordance with the whole body autoradiography pictures. They confirm that the orally administered test material can be found mainly in the organs of the gastrointestinal tract (stomach, small and large intestines), as well as in the excretory organs (liver and kidneys). The test material hardly penetrates through the blood-brain barrier. Within 24 hours after the administration, the administered test material is practically eliminated from the body.

Table 5. Organ and tissue radioactivity levels after oral administration of 10 mg/kg [^{14}C]CH-13584 to rats

Organs	RADIOACTIVITY					
	0.5 hour			3 hours		
	µgEq./g		S.D.	µgEq./g		S.D.
Brain	1.29	±	0.18	0.22	±	0.05
Lungs	6.00	±	0.58	0.72	±	0.24
Heart	5.94	±	1.05	0.67	±	0.25
Spleen	5.91	±	0.82	0.49	±	0.24
Liver	8.95	±	0.96	1.29	±	0.49
Kidneys	21.48	±	8.68	3.24	±	0.85
Adrenals	8.41	±	2.01	1.72	±	1.05
Thymus	5.82	±	0.47	0.71	±	0.22
Stomach	145.89	±	87.06	3.84	±	3.21
Small intestines	13.56	±	3.79	4.51	±	2.79
Large intestines	5.84	±	0.94	14.46	±	6.87
Sexual organs	3.50	±	0.74	0.83	±	0.34

Organs	Radioactivity					
	6 hours			24 hours		
	µgEq./g		S.D.	µgEq./g		S.D.
Brain	0.05	±	0.02	0.00	±	0.00
Lungs	0.11	±	0.04	0.02	±	0.00
Heart	0.09	±	0.02	0.00	±	0.00
Spleen	0.07	±	0.04	0.02	±	0.00
Liver	0.31	±	0.09	0.07	±	0.04
Kidneys	0.25	±	0.11	0.04	±	0.02
Adrenals	0.83	±	0.49	0.07	±	0.05
Thymus	0.09	±	0.04	0.02	±	0.00
Stomach	0.62	±	1.21	0.09	±	0.13
Small intestines	0.43	±	0.72	0.04	±	0.02
Large intestines	13.52	±	9.19	0.18	±	0.09
Sexual organs	0.11	±	0.05	0.00	±	0.00

2.3.2.2.3. Distribution of selegiline in rat brain [VI, VII]

Selegiline is a selective MAO-B inhibitor used in the treatment of Parkinson's disease, However, its additional pharmacological effects, such as inhibition of dopamine uptake, some amphetaminergic effects, neuroprotection, antiapoptopic effects, neuronal rescue effect, [VII] etc., were revealed which could hardly be explained by the MAO-B inhibitory activity. Some of them could be attributed to the metabolism of deprenyl, but some of them are still unclear. For these reasons it was important to examine in details the distribution of selegiline and its metabolites in the brain.

For the experiments, a mixture of tritium-labelled and ^{14}C-labelled selegiline preparations was used (see paragraph 2.1.1.3). The technique for the determination of radioactivity in the cerebral tissues was practically the same as described in paragraph 2.3.2.2.2. with the exception that ^{3}H and ^{14}C were measured parallelly.

The following cerebral tissues were dissected and measured from the rats treated with selegiline at the oral dose of 1.5 mg/kg: bulbus olfactorius, cerebellum, colliculus superior, corpus pineale, corpus striatum, frontal cortex, hippocampus, hypophysis, hypothalamus, medulla oblongata, nucleus mamillaris, parietal cortex, pons + colliculus inferior, substancia nigra, tuberculum olfactorium. The distribution of radioactivity in brain was tested 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 12, 24, 48, 72 and 96 hours after drug intake.

Table 6. $AUC_{0\text{-}96}$ values of cerebral tissues from rats treated orally with 1.5 mg/kg selegiline

Tissues	$AUC_{0\text{-}96}$ ^{3}H [pmol/mg/h]	$AUC_{0\text{-}96}$ ^{14}C [pmol/mg/h]	^{3}H to ^{14}C ratio [-]
Plasma	107.89	33.59	3.2
Corpus pineale	133.70	0	
Bulbus olfactorius	100.70	45.1	2.2
Hypophysis	55.25	33.27	1.7
Hypothalamus	166.31	48.92	3.4
Tuberculum olfactorium	137.32	55.13	2.5
Substancia nigra	152.56	0	
Nucleus mamillaris	116.56	0	
Frontal cortex	159.89	34.26	4.7
Parietal cortex	122.64	37.45	3.3
Corpus striatum	70.34	23.41	3.0
Hippocampus	118.62	32.18	3.7
Colliculus superior	189.71	56.31	3.4
Cerebellum	183.90	50.46	3.6
Pons+Colliculus inferior	147.08	38.92	3.8
Medulla oblongata	143.92	37.63	3.8

In all of the cerebral tissues examined, the molar concentration of ^{3}H-labelled compounds representing selegiline and its metabolites containing phenyl group (e.g. methamphetamine, amphetamine, desmethyl-selegiline, etc.) is higher than the molar concentration of ^{14}C-labelled compounds representing selegiline and its metabolites containing propargyl group (e.g. desmethyl-selegiline, propiolaldehyde, propargylamine, etc.). These results show that considerable quantity of metabolites are formed, already by "first pass" metabolism, and N-despropargyl derivatives of selegiline are dominant in the cerebral tissues.

2.3.2.2.4. In vitro and *in vivo* binding of CH-13584 to serum (plasma) proteins [XV]

Using an Amicon 8MC ultrafiltration system and ^{14}C-labelled CH-13584 preparation, protein binding of CH-13584 was studied by simple ultrafiltration technique, *in vitro* and *in vivo* in rats, and *in vitro* in human.

In the *in vitro* experiment a concentration series of 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5, 25 and 50 mg/L CH-13584 was applied. The results of the protein binding study can be seen in Table 7. The results show that no significant protein binding of CH-13584 occurs in rat and human plasma *in vitro*.

For the *in vivo* study, Wistar rats were administered with 10 mg/kg oral dose of [^{14}C]CH-13584, and blood samples were taken 1 and 3 hours after dosing. Plasma was obtained by centrifugation, and the same equipment was used for the determination of the binding to plasma proteins. The average plasma concentrations were 3.5 ± 0.3 and 0.3 ± 0.1 mgEq./L 1 and 3 hours after the treatment, respectively. The unbound fraction was 86.5 ± 1.5 and 71.3 ± 6.8 % on the average. The results suggest that the metabolites formed from CH-13584 are bound to plasma proteins at a somewhat higher percentage than the parent compound, but this level of binding is practically still not significant.

Table 7. *In vitro* binding of [^{14}C]CH-13584 to rat and human plasma proteins

Concentration of [^{14}C]CH-13584 [mg/L]	**Unbound fraction**	
	Rat plasma %	**Human plasma %**
0.010	100.31	90.12
0.025	90.19	94.50
0.050	90.43	90.96
0.100	88.77	93.34
0.250	84.66	90.09
0.50	86.05	95.90
1.00	87.21	93.24
2.50	88.96	91.26
5.0	86.95	92.97
25.0	83.89	88.20
50.0	86.18	87.45

2.3.2.3. Excretion

For safety reasons, it is very important that the test material is eliminated from the body within a certain period. In any case, a drug have to be excreted after exerting its effect, otherwise it is impossible, or at least very difficult to regulate its action. A drug having long-lasting elimination can have long-lasting pharmacological effect, too, but it also can be cumulated in the body, especially in an organ, and it can cause unwanted side effects.
Not only the rate but also the route of the excretion is important. The two main routes are the urine, through the kidneys, and the bile, through the liver. The drug or its metabolites excreted in bile can be eliminated via feces, or can be reabsorbed from the intestines (entero-hepatic circulation).
There are some other routes for the elimination of a drug, such as the sweat, saliva, breast milk, expired air, but generally, these routes are not significant. However, one of these routes, namely the expired air is studied very frequently. The radioactive test material — following a metabolic reaction — can loose the [^{14}C] in the form of [^{14}C]carbon dioxide, and then not the test material or its metabolites, but only a small fragment of the molecule is monitored. Practically, the measurement of radioactivity in the expired air is a kind of control of the synthesis of the radiolabelled material. If significant amount of radioactivity can be found in expired air, the position of the radioactive nuclide was not appropriately designed.

2.3.2.3.1. Mass balance of [^{14}C]CH-13584 in rats [XIV]

Urine, feces and expired air were collected in 24-hour fractions from male and female rats treated orally with 10 mg/kg of [^{14}C]CH-13584. Radioactivity of the samples was measured by liquid scintillation counting. Practically no radioactivity could be detected in the expired air; less than 0.01 % of the dose could be found within 168 hours.
The mass balance of [^{14}C]CH-13584 administered to rats can be seen in Figure 19. and Figure 20. The majority of the radioactivity was excreted with urine; within 168 hours, 87.12 ± 8.43 and 78.64 ± 8.34 % of the dose could be recovered in the urine in males and females, respectively. Within the same period, the fecal elimination of the radioactivity was 13.24 ± 7.70 and 11.17 ± 4.84 % of the dose, thus the total recovery was 98.31 ± 6.06 and 91.91 ± 2.46 % of the dose in males and females, respectively.

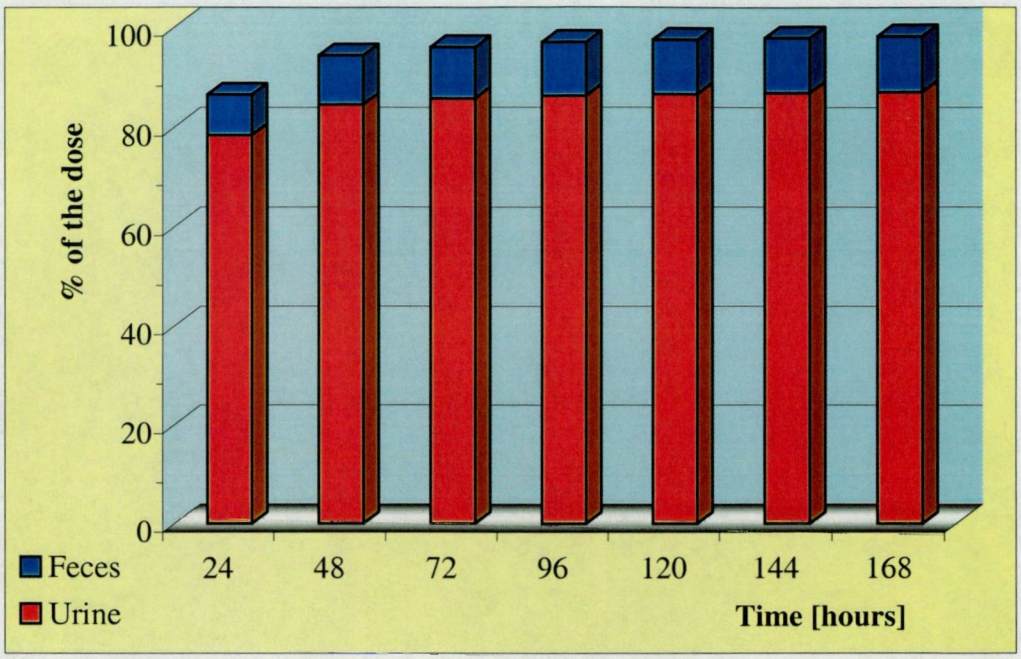


Figure 19. Urinary and fecal elimination of radioactivity after single oral administration of [^{14}C]CH-13584 to male rats

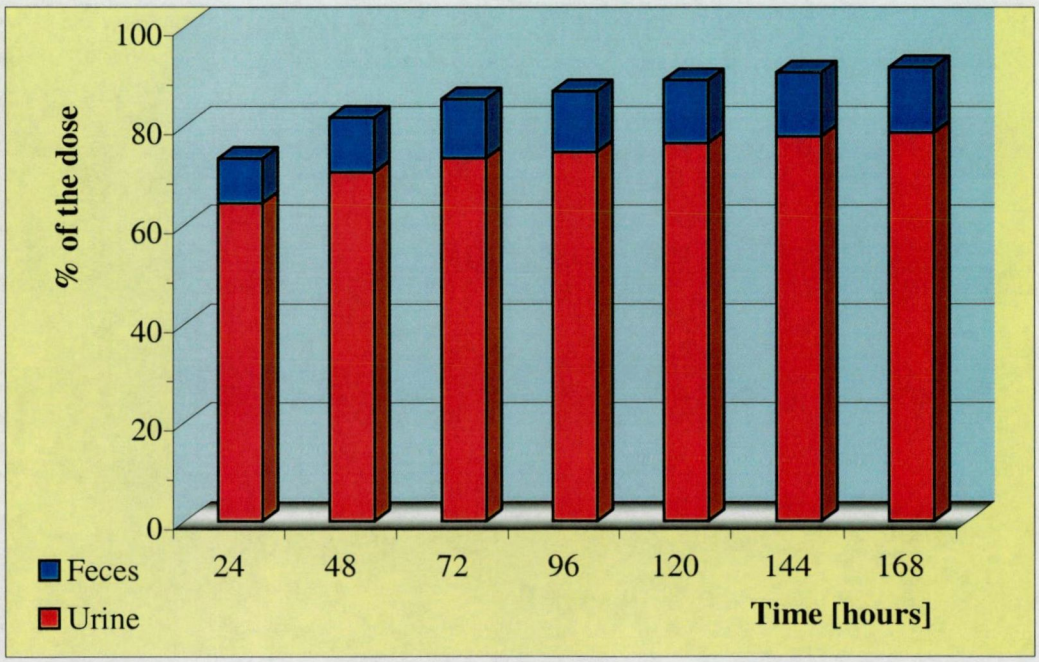


Figure 20. Urinary and fecal elimination of radioactivity after single oral administration of [^{14}C]CH-13584 to female rats

2.3.2.3.2. Urinary elimination of [^{14}C]DIMEB in rats [IV]

There are certain cases, when the method used regularly for studying urinary elimination of a test material is not appropriate to characterize its pharmacokinetic features. It was the case, when pharmacokinetics of heptakis-(2,6-di-*O*-methyl)-β-cyclodextrin (DIMEB), a potential agent for increasing the solubility of poorly water soluble drugs by forming inclusion complexes, were studied.

Since some cyclodextrins may have tissue-damaging effect, it was crucial to demonstrate that DIMEB has a very short residence time in the body. Preliminary results showed, that in rats, 80-90 % of the dose was eliminated within 72 hours in urine after a single intravenous administration of [^{14}C]DIMEB.

Continuous urine collection, or serial collection of urine in short time intervals is relatively easy in human and in bigger laboratory animals such as dogs, using a catheter. The technique, however, was not developed for small laboratory animals, and especially for rats. Using a new surgical technique, the collection of urine samples in one-hour fractions from not anaesthetized, unrestricted rats was performed [IV]. Analyzing the fractions, the very rapid excretion of DIMEB was proven from the systemic circulation into urine. More than 50 % of the dose was found in the first (0-1 hour) urine fraction and more than 70 % was excreted within 3 hours (Figure 21.).

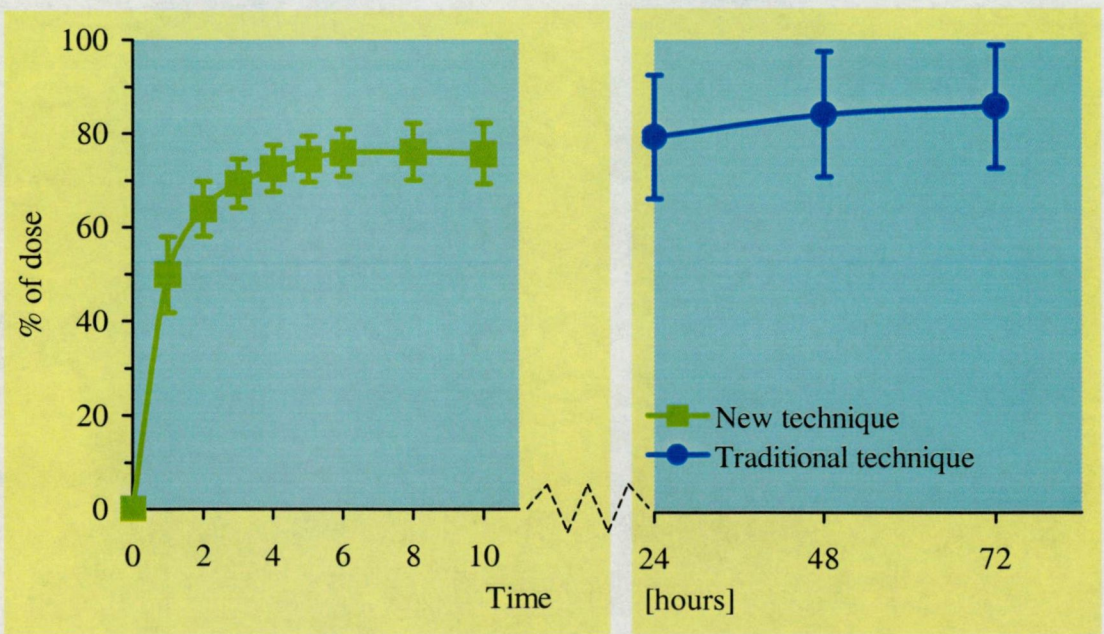


Figure 21. Urinary elimination of DIMEB in rats after intravenous administration

2.3.3. Metabolism

Metabolism of drugs and the role of liver microsomal enzymes have been already discussed in paragraph 2.2.3.

The metabolic steps can be divided into two main groups. By the **phase 1** metabolic reactions, which are generally oxidative reactions, the chemical structure of the lipophilic drugs is modified to form biotransformation products (metabolites), which are more polar, more

hydrophylic compounds. The most common reactions are oxidation, hydroxylation and the oxidative *N*- or *O*-dealkylation. During metabolic processes, the oxidative reactions are usually followed by conjugation reactions resulting in polar conjugates that are easily excreted. The most common conjugation reaction (**phase 2** metabolic reaction) is catalyzed by UDP-glucuronyltransferases. Beside these glucuronide conjugates, formation of glutathion-, sulphate- and methyl conjugates can also be observed.
The aim of the metabolism studies in the preclinical development phase of the drug is to determine the main metabolic routes by identifying the chemical structure of the metabolites. Some years ago, the separation (TLC, HPLC, column chromatography) and isolation (scrapping from TLC, fraction collection after HPLC or column chromatography) of the metabolites were necessary for the identification of the chemical structure using MS, NMR, IR and/or Raman spectroscopy. Nowadays, when LC-MS/MS and LC-NMR equipment are available, the structure elucidation of the metabolites is much less time-consuming. Nevertheless, there are cases when new metabolites are identified many years after a drug is launched. It must be noted here, that a metabolite can be considered as identified if it was synthesized, and the chromatographic, mass spectrometric and NMR characteristics of the synthesized reference standard are identical with those of the metabolite.

2.3.3.1. Metabolism of CH-13584 in rat, dog and guinea pig [XII, XVII]

Metabolism of CH-13584 was investigated in rats, dogs and guinea pigs. Practically, only unchanged CH-13584 could be detected in plasma in all animal species studied. Urine samples were analyzed by HPLC, and twelve metabolites were found in the chromatograms. The ratio of the metabolites showed considerable individual variation, but it also showed significant sex difference in the case of dogs (Table 8). Except female dogs, unchanged CH-13584 was found mainly in the urine; an average of 68.7, 94.0 and 74.8 % of the total metabolites excreted in urine was found unchanged in rats, guinea pigs and male dogs, respectively. Beside the parent drug, M3, M4, M5 and M10 metabolites represented considerable amounts. In the case of female dogs, M4 was the main urinary metabolite.
Out of the twelve metabolites, the chemical structure of four ones could be identified, and the chemical structure of one metabolite could be suspected [XVII].
Accordingly, M11 was found to be the unchanged CH-13584, M5 was a metabolite where the oxadiazole ring was opened (3-methyl-7-acetamidoxanthine), M6 was identified as 3-methylxanthine, M8 was found to be the 3-desmethylated derivative of CH-13584, and M4 was supposed to be xanthine. The metabolic pathways are demonstrated in Figure 22.

Table 8. Elimination of CH-13584 and its metabolites in rat, dog and guinea pig urine (Relative %)

Metabolites (M)	Rats (N=6)		Guinea pigs (N=2)		Dogs			
					Males (N=3)		Females (N=3)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1-2	0.0	0.0	0.0	0.0	0.3	0.3	1.0	0.9
3	4.7	9.2	0.3	0.0	1.5	1.4	19.1	28.4
4	14.0	20.0	0.4	0.3	11.6	7.9	32.2	14.8
5	4.4	1.9	0.3	0.2	5.2	0.2	17.4	6.8
6	1.3	0.6	0.2	0.0	0.5	0.1	0.0	0.0
7	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.1
8	0.2	0.3	0.1	0.2	5.5	3.4	0.8	1.1
9	1.1	1.9	4.0	0.0	0.3	0.3	0.0	0.1
10	4.6	7.3	0.0	0.0	0.0	0.0	8.8	8.3
11	68.7	27.1	94.0	0.2	74.8	5.9	14.7	16.1
12	1.0	2.0	0.4	0.1	0.3	0.3	6.0	8.8

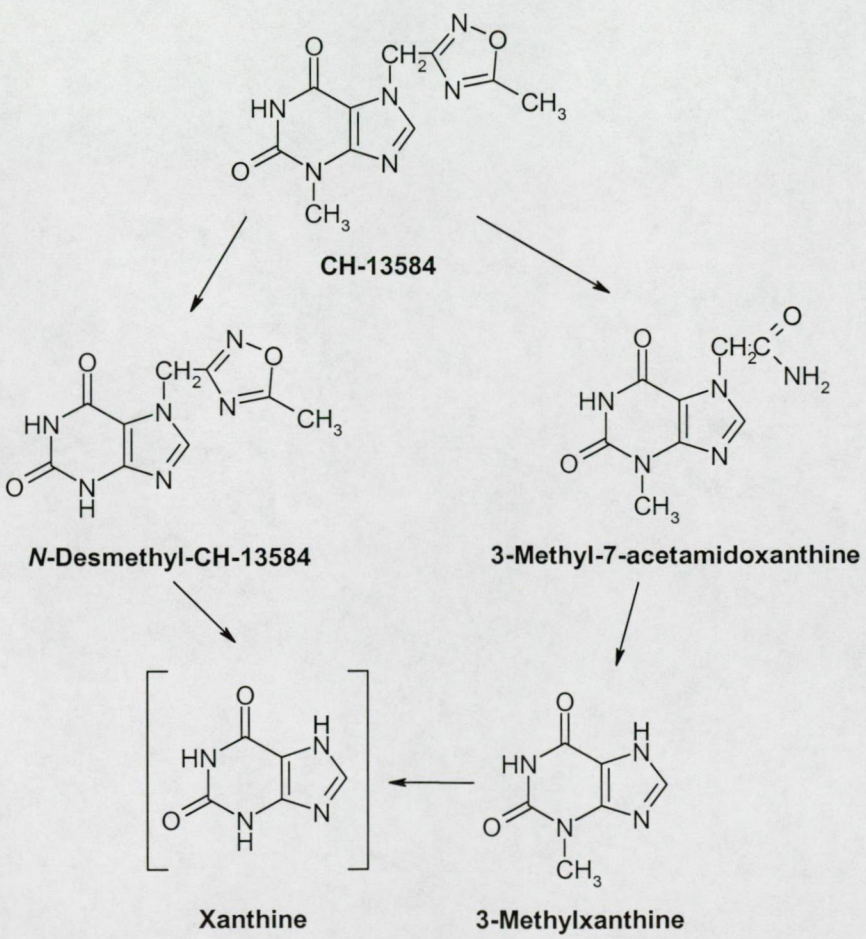


Figure 22. Metabolic pathways and structure of the identified metabolites of CH-13584

2.3.3.2. Metabolism of selegiline [V, XI]

CH-13584 is a drug, which is rather stable in the organisms, and which has relatively simple metabolism pathway. The majority of the compounds is not as such.

Selegiline (also called as (-)-deprenyl, or L-deprenyl), chemically (-)-*N*,α-dimethyl-*N*-2-propynylbenzeneethanamine, is a selective and irreversible inhibitor of monoamine oxidase type B. It is widely used in the treatment of Parkinson's disease. Selegiline is an original CHINOIN drug, however, its pharmacology, pharmacokinetics and metabolism, as well as its clinical characterization were mainly studied out of CHINOIN, generally on a contract basis, or by the license partners.

Although racemic selegiline was discovered in 1962[29] and its optical isomers were synthesized and patented in the middle of the sixties[30], the first pharmacokinetic and metabolism results were published only six-ten years later [XI]. The main metabolites of selegiline, such as desmethylselegiline, methamphetamine and amphetamine were discovered very early, while minor metabolites were identified much later. Because of the success of selegiline, and because of its unique pharmacology, after almost forty years, studies are still being performed; newer and newer clinical advantages are recognized; new minor metabolites are identified. Among the metabolites of selegiline, *p*-hydroxyl-desmethylselegiline, *p*-hydroxyl-amphetamine, *p*-hydroxyl-methamphetamine, *N*-acetylamphetamine, norephedrine and nor-pseudoephedrine, ephedrine and pseudoephedrine, phenylacetone and phenylacetone-oxime were identified as minor ones in animal species or in human. Selegiline-*N*-oxide could be recently identified as a metabolite of selegiline (see paragraph 2.3.3.2.1). The metabolic pathways of selegiline, as well as its putative metabolites supposed on theory or analogy can be seen in Figure 23.

2.3.3.2.1. Identification of selegiline-*N*-oxide as a metabolite of selegiline [XIX, XX]

Based on analogy, the formation of selegiline-*N*-oxide was supposed already in the eighties[31,32]. Later, when pharmacokinetic and metabolic characterization of selegiline was performed using a quasi-double-labelled radioactive preparation (see paragraph 2.1.1.3), we found an unknown metabolite, which contained both the propargyl and the "amphetamine" parts. However, when the metabolite was injected into the gas chromatograph, it always underwent a decomposition process forming selegiline. Then, we had the hypothesis that the unknown metabolite was selegiline-*N*-oxide. Chemists of CHINOIN Preclinical Development synthesized selegiline-*N*-oxide (both *R,R* and *R,S* diastereomers, because stereoselective N-oxidation was published for pargyline, a chemically very similar MAO inhibitor[33]), and thus, selegiline-*N*-oxide could be identified as a metabolite of selegiline. The formation of selegiline-*N*-oxide in liver microsomal preparations obtained from different animal and human species shows rather big differences. While significant amount of selegiline-*N*-oxide was found in dog and hamster microsomes, less amount was detected in mice, rats, guinea pigs and rabbits, and only very little selegiline was metabolized to its *N*-oxide derivative in human preparation (Table 9.).

In microsomal preparations obtained from animal and human species studied, except of hamster, the *in vitro* formation of the *R,S* diastereomer of selegiline-*N*-oxide was preferred (57.7-81.8%).

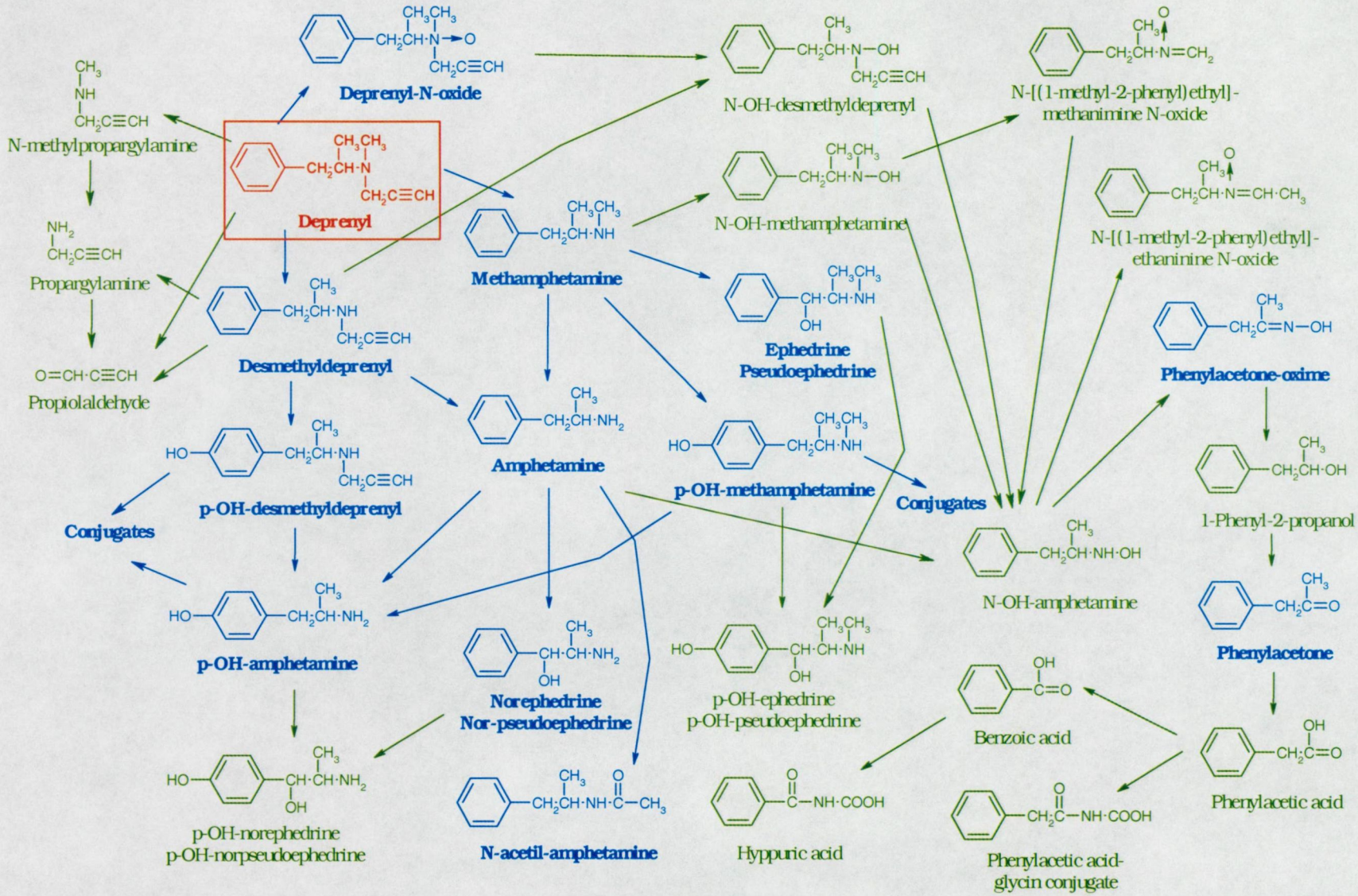


Figure 23. Metabolites of selegiline identified in animal species and in human (bold blue), as well as its putative metabolite pathway supposed on theory or analogy (green) [XI]

Table 9 The ratio of the metabolites of selegiline after incubation in liver microsomal preparations from different species (in % of peak area of selegiline measured in 0 minute-samples)

Species	AMP	MET	DES	SEL	SNO	TOT
Rat	12.5	27.7	21.4	0.6	**20.2**	82.4
Dog	6.3	25.4	2.6	0.0	**55.6**	89.8
Hamster	15.5	2.4	0.0	0.0	**41.2**	59.1
Rabbit	14.7	9.5	12.1	20.2	**14.4**	71.0
Mouse	13.0	28.9	20.1	3.9	**21.5**	87.5
Guinea pig	24.0	42.2	0.0	0.0	**19.8**	86.0
Human	0.0	15.2	9.9	74.6	**1.0**	100.6

AMP=amphetamine, MET=methamphetamine, DES=desmethylselegiline, SEL=selegiline, SNO=selegiline-*N*-oxide, TOT=total recovery as "main" metabolites

2.4. CLINICAL PHARMACOKINETICS AND METABOLISM

The aim of the clinical pharmacokinetic and metabolism studies is to clarify if the drug, which was already examined in non-clinical systems in details, is absorbed, reaches a certain level in the plasma, is eliminated within a certain time, if it is safe to use in men. The majority of the pharmacokinetic and metabolism methods used in the clinical studies are similar to those used in the preclinical ones. In fact, the pharmacokinetic evaluations are based on bioanalytical measurements of the unchanged compound and its metabolites in plasma, urine and feces samples obtained from subjects treated with the drug.

Among the clinical pharmacokinetic and metabolism studies, single and multiple dose tolerance studies (Phase 1), single and multiple dose pharmacokinetic studies, mass balance and metabolite identification studies (Phase 2), pharmacokinetics in patients with renal or liver disease, formulation bioavailability, drug-drug interaction studies can be mentioned. However, depending on the drugs studied, or on the therapeutic field aimed, other investigations can also be performed, or some of the studies can be carried out within one study.

2.4.1. Human pharmacokinetics of drotaverine-acephyllinate [III]

Drotaverine-acephyllinate[34] (also called as Depogen®), is a drug having peripheral vasodilator and spasmolytic effects, similarly to NoSpa®, a core product of CHINOIN. In fact, Depogen® is the theophylline-7-acetic acid salt of drotaverine (Figure 15.). Its pharmacokinetic

characteristics were studied in seven male healthy volunteers, using ^{14}C-labelled preparation, where the ^{14}C label was in the drotaverine part of the molecule. The applied dose was 100 mg in a hard gelatine capsule.

Figure 24. shows the radioactivity concentrations in blood and plasma, while Figure 25. demonstrates the radioactivity and unchanged drotaverine concentrations in plasma.

The results showed that peak plasma concentration was reached between 0.75 and 1 hour after drug administration. Plasma and blood radioactivity curves showed an almost identical course. The unchanged drug and its metabolites were not bound to blood cells.

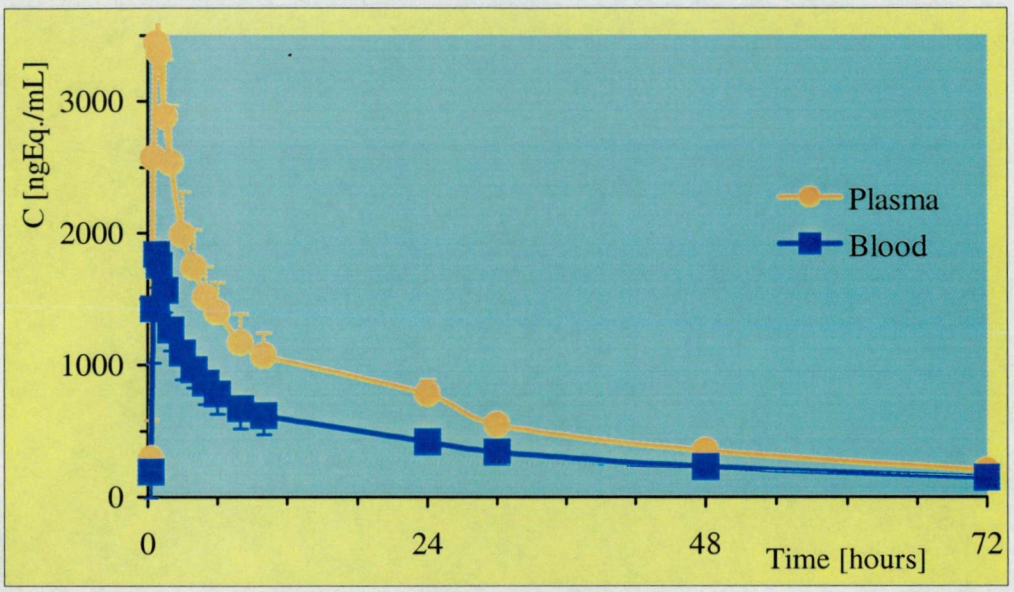


Figure 24. Average plasma and blood radioactivity concentrations in men after single oral administration of [^{14}C]drotaverine acephyllinate

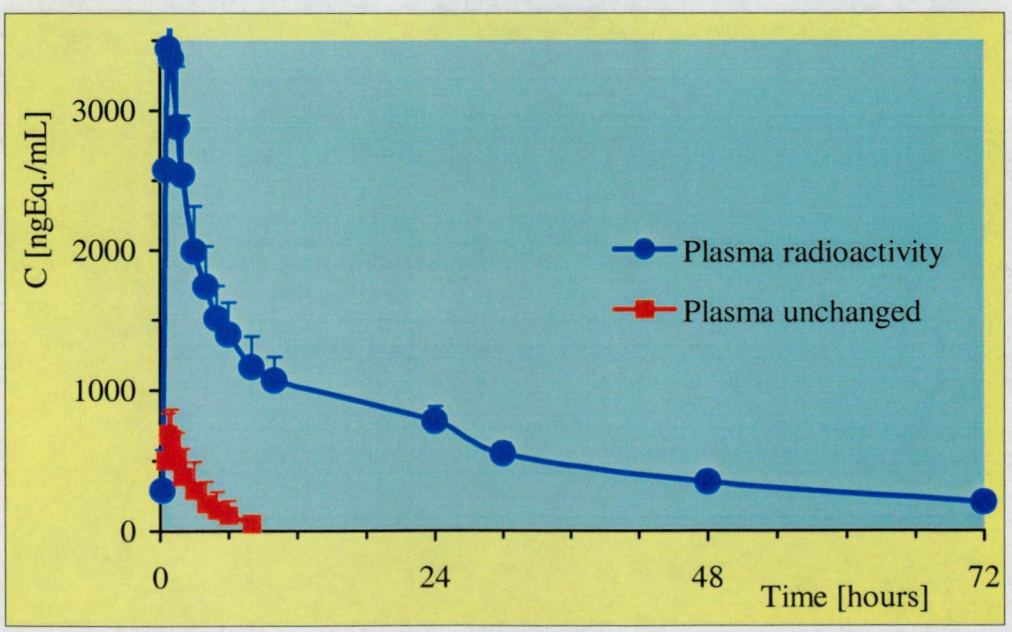


Figure 25. Average radioactive and unchanged drotaverine concentrations in human plasma after single oral administration of [^{14}C]drotaverine acephyllinate

The parent drug concentrations were about one-fifth of the total radioactivity concentrations. Drotaverine part of Depogen® was almost completely metabolized by *O*-desethylation to mono- and diphenolic moieties, and these metabolites were rapidly conjugated with glucuronic acid.
As in can be seen in Figure 26., almost equal portions of the administered dose were eliminated in urine and feces (39.0 ± 9.9 and 47.1 ± 4.9 % of dose, respectively, within 72 hours). The total recovery within 72 hours was 86.1 ± 10.1 % of the administered dose.

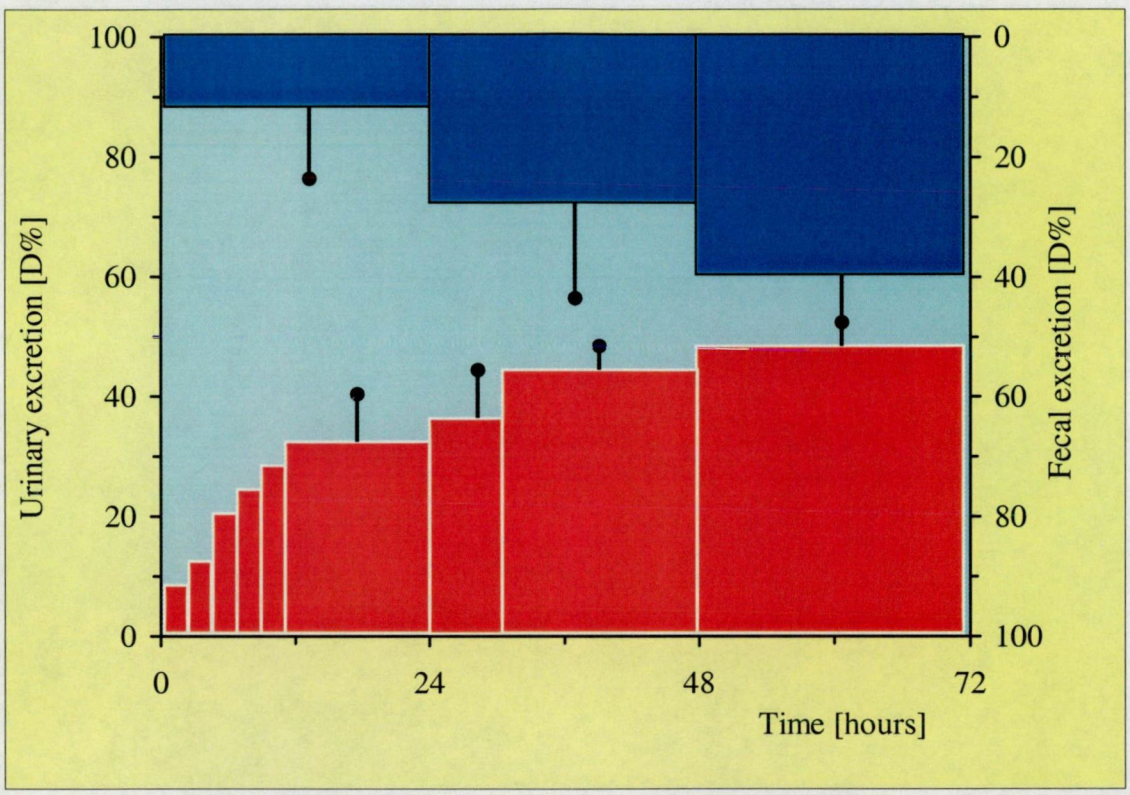


Figure 26. Cumulative urinary and fecal elimination of radioactivity after single oral administration of [^{14}C]drotaverine acephyllinate

2.5. INTERACTION STUDIES [VIII]

As it was discussed earlier (2.2.3), CYP450 enzymes play an important role in the biotransformation of lipophilic drugs. Any changes in the activity of CYP450 isoenzymes may influence the rate of activation (if the metabolite is more active than the parent compound) or inactivation (if the metabolite is less active than the parent compound) of drugs. Consequently, if a drug induces or inhibits one or more CYP isoenzymes, it can modify the pharmacological and/or the safety profile of another drug metabolized by the affected isoform. It must be mentioned that pharmacokinetic interactions cannot exclusively be related to metabolism, but also to protein binding, absorption or elimination.
Ipriflavone (also called as OSTEOCHIN®), a synthetic flavonoid, was developed for treatment of osteoporosis. In the case of a Japanese patient, who took regularly theophylline for chronic obstructive pulmonary disease, an increase in the theophylline serum concentration was

observed when ipriflavone was concomitantly administered. After withdrawal of ipriflavone, theophylline serum level was decreased. It was clear that metabolism of theophylline was altered by ipriflavone[35].

In an *in vitro* interaction study, we examined which CYP450 isoenzymes could be responsible for the *in vivo* interaction of theophylline and ipriflavone [VIII].

Using human liver microsomal preparation, the effect of ipriflavone and its two main human metabolites, 7-hydroxyisoflavone and 7-(1-carboxyethoxy)isoflavone, on the activity of CYP450 isoenzymes, such as CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 was examined. The isoenzyme activities were monitored by measuring the metabolism of selective substrates of the isoenzymes. Inhibitory effect of ipriflavone and its two main metabolites were expressed as the percentage of the control incubation (without ipriflavone or metabolite).

The results showed that ipriflavone and 7-hydroxyisoflavone had inhibitory effects on phenacetine O-desethylase (CYP1A2) and tolbutamide hydroxylase (CYP2C9), while the main human metabolite, 7-(1-carboxyethoxy)isoflavone did not alter the activities of the CYP450s investigated. Since CYP2C9 is not an isoenzyme, which catalyzes any step of theophylline metabolism, it cannot be responsible for the interaction between ipriflavone and theophylline. However, CYP1A enzymes are known to be involved in the metabolism of methylxanthines, especially in their *N*-demethylation. Thus, it can be supposed that — in the case of the Japanese patient mentioned above[35] — ipriflavone and one of its main metabolites, 7-hydroxyisoflavone, inhibited *N*-demethylation of theophylline through CYP1A2 inhibition. It must be noted, that no other case of interaction caused by ipriflavone was reported.

2.6. DEVELOPMENT OF NEW FORMULATIONS [X]

An appropriate formulation can change not only the physical but also the pharmacokinetic or therapeutic characteristics of a drug. It can increase the bioavailability (e.g. by increasing solubility) of a drug[36,37]; it can provide the controlled release of the active ingredient (once-a-day formulations)[9]. Sometimes the new formulation also means new administration route, at the same time, for example, preparation of suppositories or transdermal[38,39] formulations is rather common in the pharmaceutical industry. This was the case in CHINOIN with selegiline, a drug used successfully in the Parkinson's disease[40,41] [X].

From simple ointments through transdermal silicon patch, to liposome formulations [X], several transdermal formulations were prepared and examined. Moreover, a new two-phase formulation — based on the mechanism of action of selegiline — was also developed[42].

The pharmacokinetic effort in these studies always had two targets. On one hand, the pharmacokinetic profile requested should be tested; on the other hand, the bioavailability of the active ingredient had to be determined in different species. Sometimes, when the equivalence of the "old" and "new" formulation was declared, bioequivalence studies were performed.

3. SUMMARY

In the last ten-fifteen years, pharmacokinetic and metabolism studies became essential part of the drug research and development; they overlap the discovery, preclinical and clinical phases. Early in the nineties, we developed a system for pharmacokinetic and metabolism studies, based on regulations, guidelines, international tendencies, traditions and capabilities. The system described the pharmacokinetic and metabolism studies to be performed in the discovery phase (Figure 27.), and we also put a screen into practice (*in vitro* absorption, *in vitro* metabolism), in order to help the selection of new drug candidates. The system involved the pharmacokinetic and metabolism studies to be performed in the preclinical development phase (Figure 28.). We established an animal experimental unit, where studies required for the registration of the drug can be performed according to the standards. The laboratory got a certificate from the Hungarian registration authority (OGYI) in 1997, which certificate declares the compliance of the laboratory with the principle of the Good Laboratory Practice (GLP). The system also included the pharmacokinetic and metabolism studies to be performed in the clinical development phase (Figure 29.), and we could implement a bioanalytical background with bioanalytical specialists and equipment, thus we have a facility in compliance with the principles of Good Clinical Practice (GCP).
So the main result of my activity described in this paper, that there is a working system, a well-operating organization in CHINOIN R&D, with thirteen people at this time. Colleagues can perform practically all kind of pharmacokinetic studies required from the very beginning of drug discovery up to the registration of a new drug, and beyond. We recognized that to characterize a new drug for the pharmacokinetics and metabolism is always a scientific novelty.
In this paper, I showed selected examples on the studies performed with compounds developed in CHINOIN. Some of them were performed earlier than the new system was established, but the majority was carried out in the last ten years. For that very reason, these selected examples are excellently suitable to demonstrate the change in the pharmacokinetic and metabolism approach carried out in CHINOIN R&D in accordance with the international practice. One of the main aims of this paper was to show how this change in the paradigm appears in the daily work.

- I stressed the importance of the bioanalytical activity, including the separation techniques and the use of radiolabelled materials.
- I introduced the *in vitro* absorption and metabolism screens, and how these screens can help the drug discovery. I proved the importance of these screens in the selection of the pharmacological species. I demonstrated the chemical structure and metabolic stability relationship through the example of prolylendopeptidase inhibitors.
- I presented results on the preclinical pharmacokinetic and metabolism of CH-13584, an original CHINOIN antitussive agent:
  - I proved that CHINOIN-13584 is absorbed orally in rats, however, at very high doses, the absorption is not dose-proportional.
  - I demonstrated that CH-13584 is distributed rapidly, but it cannot pass the blood-brain barrier.
  - I presented that the drug and its metabolites are eliminated mainly in urine, and the recovery of the test material is practically complete within 168 hours.

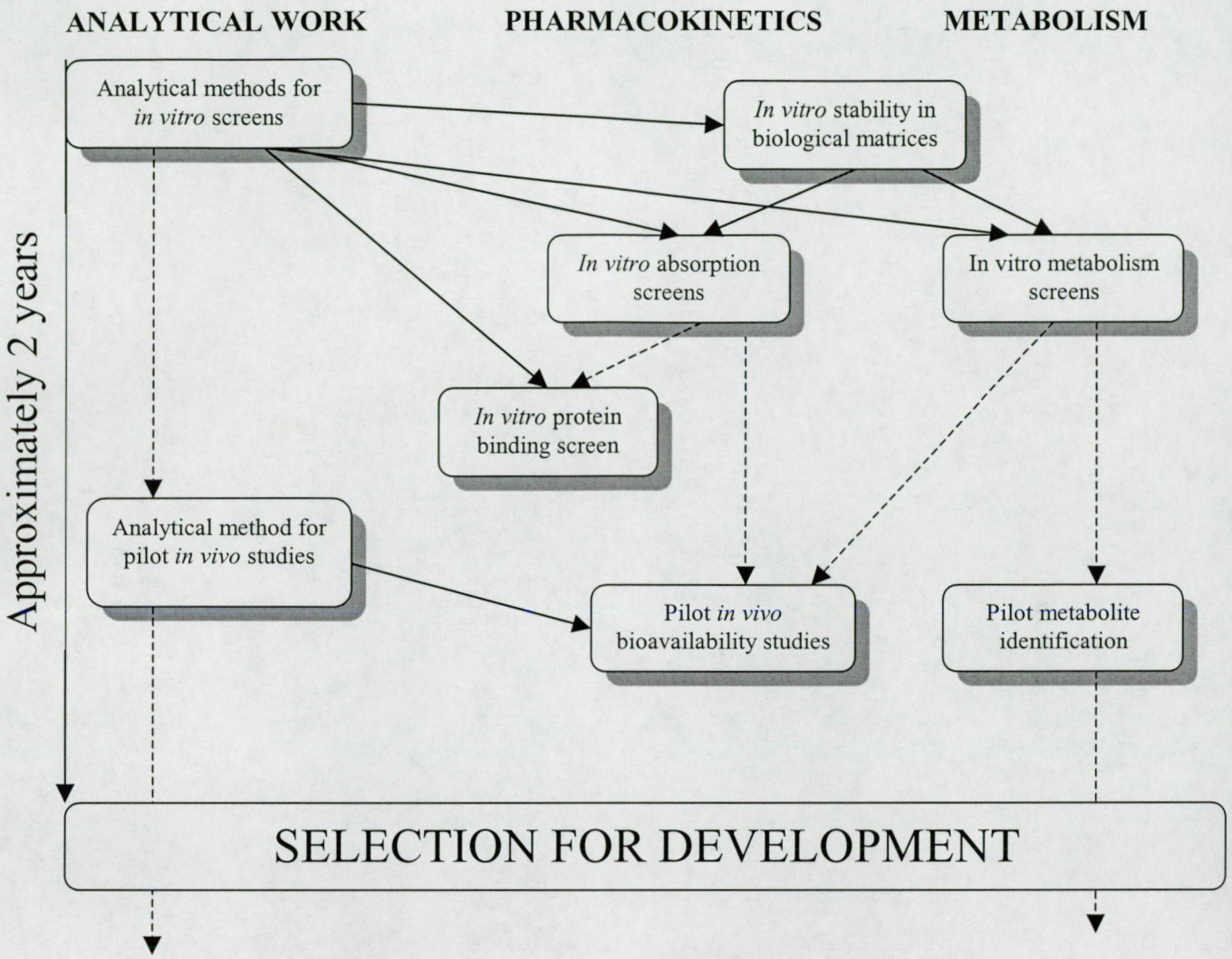


Figure 27. Pharmacokinetic and metabolism studies to be performed during the discovery phase of drug research and development

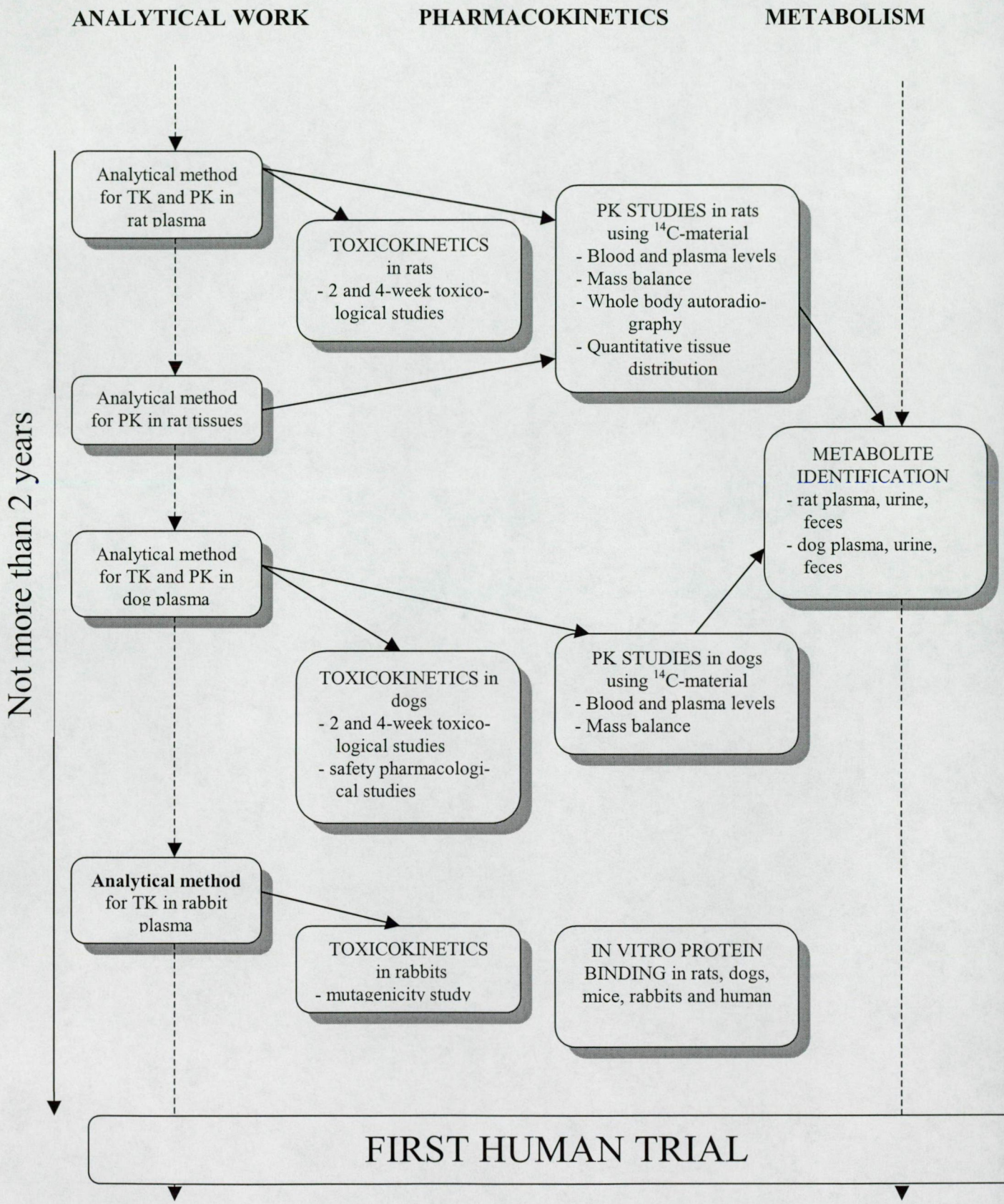


Figure 28. Pharmacokinetic and metabolism studies to be performed during the preclinical development of drug research and development

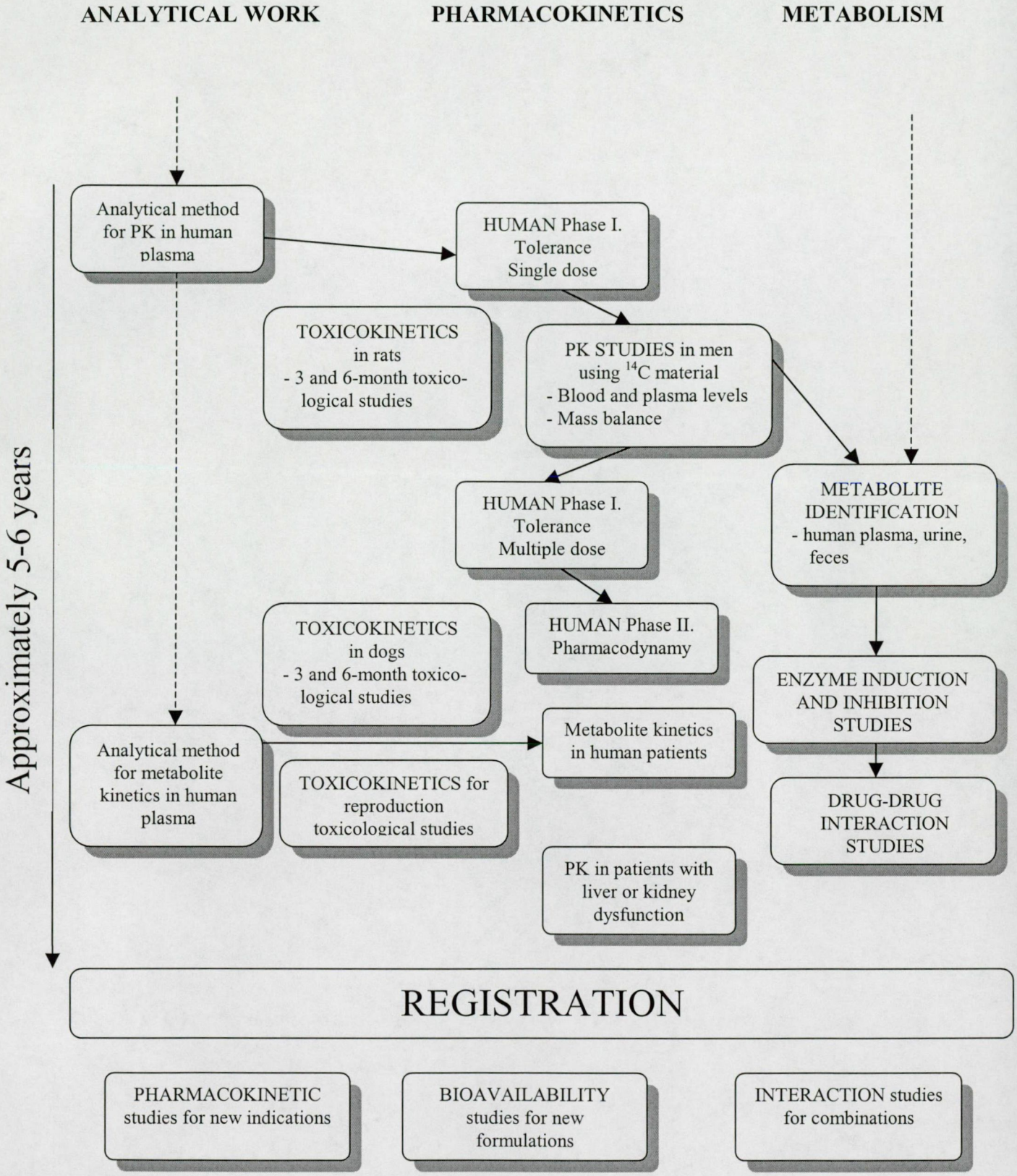


Figure 29. Pharmacokinetic and metabolism studies to be performed during the clinical development of drug research and development, as well as after registration

- I showed that CH-13584 is poorly metabolized in several animal species; the metabolic pathways are the *N*-demethylation and the oxidation of the oxadiazole ring.
- I demonstrated that binding of CH-13584 to plasma proteins is negligible.

- As I mentioned earlier, special studies have to be performed sometimes, to characterize the test material better. I presented some results obtained from such kind of studies:
  - I presented that drotaverine acephyllinate (Depogen®) is rapidly absorbed from the duodenal and ileal parts of the small intestines. I obtained the results from a study using *in vivo* loop technique.
  - I proved that dimethyl-β-cyclodextrin (DIMEB) is eliminated very quickly from the body via urine. For this study, I developed and applied a rather new surgical technique, which was appropriate for gathering urine samples from unanaesthetized, unrestricted rats in short (1-hour) time fractions.
  - I proved that selegiline metabolites lost propargyl group are dominant in the rat brain after oral administration of selegiline. By this, I showed that beside selegiline, selegiline metabolites play a significant role in the pharmacological effect of the drug.
  - I demonstrated that the metabolic pathways of a drug can be very diverse, and the identification of the metabolites sometimes takes rather long time. I proved that selegiline-*N*-oxide is a metabolite of selegiline, which was discovered twenty years ago, but the chemical structure could be only recently identified.
- The results of the clinical pharmacokinetic and metabolism studies of Depogen® are as follows:
  - I proved that the drotaverine part of Depogen® is absorbed very quickly, and the peak plasma concentration is reached between 0.75 and 1 hour after oral administration of the ^{14}C-labelled preparation. I demonstrated that the unchanged drug and its metabolites are not bound to blood cells.
  - I presented that one-fifth of the total radioactivity can be found in plasma as unchanged drotaverine, because it is almost completely metabolized by *O*-desethylation to mono- and diphenolic compounds, and these metabolites are conjugated rapidly with glucuronic acid.
  - I showed that almost equal portions of the administered drotaverine acephyllinate are eliminated in urine and feces, and the total recovery amounts to more than 85 % of the dose within 72 hours.
- Among the studies performed with the drugs being on the market, I presented the interaction studies between ipriflavone and theophylline, and the formulation studies with selegiline:
  - I proved that ipriflavone, a synthetic flavonoid, and one of its main human metabolites can inhibit CYP 450 1A2 and 2C9 isoenzymes.
  - Within the life-cycle management of selegiline (JUMEX®), a core product of CHINOIN, different types of transdermal formulations, such as ointments, silicon patches, liposomal formulations, as well as a special two-phase formulation were developed and characterized.

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5. ANNEX

Full papers