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MEDICINE AND PHARMACY"IULIU HAŢIEGANU" CLUJ NAPOCA UNIVERSITY

BIOCHEMICAL AND PHARMACOKINETIC STUDIES OF SOME NONSTEROIDAL ANTIINFLAMATORY DRUGS

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IBUPROFEN; KETOROLAC; ENANTIOMER; ENANTIOSELECTIVE; CHIRAL; PLASMA ; LIPASE; PHARMACOKINETICS; COMPARTMENTAL ; BIOAVAILABILITY

A.1 GENERAL ASPECTS

Chirality is an universal characteristic of the nature; therefore immediately after discovering this truth and after understanding of its involvement in the living bodies functioning, the humans tried to copy and create new molecules, with similar complexity, capable to interact as specific with the living nature.

It was very clear from the beginning that the microorganisms are capable to produce in the simplest and the most efficient way these desired molecules.

The first observation of enantioselectivity was done by Louis Pasteur who discovered in 1848 that in an aqueous solution of ammonium tartrate racemic, enriched with nutrients, the dextrogir enantiomer is mainly observed. Subsequently, he explained this by the specific action of one fungus over this enantiomer *Penicilliun glaucum*. He established the theory, that was further proved to be true, that this stereoisomer interact specific with a key molecule, chiral one, produced by the microorganism, that makes possible its transformation.[1]

Only at the beginning of the XXth century it was revealed and demonstrated the role of chirality in the action mode of the drugs. In 1933, Easson and Stedman proposed a model for enantiospecific interaction of drugs with pharmacological receptors, studying the pressor effect of epinephrine, whose enantioselectivity is 300:1, the R enantiomer being the most potent one.

The role of chirality in the chiral drugs bioavailability was further revealed, regarding pharmacological effects and chemical structures. In 1940 were studied from this point of view the sexual hormones[2], then the propranolol and other compounds with betablocant activity.[3] It was revealed the selective toxicity of the enantiomers. In 1960, during a clinical study with racemic of some anti-parkinson drug, an unacceptable toxicity of enantiomer D was discovered and the drug was further developed in enantiopure form L.[4] Numerous studies and many information regarding the importance of chirality in the metabolism of many drugs.[5]

The modern pharmacology has developed in the twentieth century. Several natural compounds were isolated and identified, thousands of new compounds were prepared, both natural and (semi) synthetic most of them chiral and an impressive number of drugs was introduced in the modern therapy.

Chiral drugs formed an increasingly important segment, in time. However, in 1987 88% of them were still used as a racemic mixture. The usage of the pharmacologically active enantiomer was achieved only when the other enantiomer present a significant toxicity. An important role in this phenomenon have been played by the pharmaceutical companies, that having no interest didn't made any effort to develop individual enantiomers of the drug whose patent they were holding.

A different attitude regarding the role of chirality in developing new drugs still started in the 8th decade of the last century, in the same time with the development of the enanatioselective analytical technique[6], this permitted the preparation of stereoselective synthesis method.[7,8]

In now days, it is mandatory to obtain the individual enantiomers of each new drug and to test its pharmacological properties, therapeutic effect and toxicity. After this a complex analysis of each enantiomer is done and the optimum decision is taken. The enantiospecific analysis is mandatory for the situation when the metabolism of the enantiomers is done by different enzymes, either polymorphic, either can be induced or inhibit and especially when the pharmacodynamics of the enantiomers is different. [9]

A new concept was introduced by Tolbert 30 years ago: usage of a non-racemic mixture of stereoisomers as an optimised drug. After studying the diuretic and uricozuric effect of indacrinonei, the optimum pharmaceutical form was established as a 4:1 mixture of the enantiomers S/R. [10]

The non-steroidal anti-inflammatory drugs are presented in a high structural variety. Despite this structural diversity, there is a distinct subclass of them: the nonsteroidal anti-inflammatory drugs with chiral structure. It is about the molecules with structure derivate from 2-arylpropionic acid (Ibuprofen, Ketoprofen, Flurbiprofen, Fenoprofen, Naproxen,Flunoxaprofen) or with arylalkanoic acid structure (Indobufen, Ketorolac, Etodolac, Clidanac) and non-acid agents (Azapropazone, Oxifenbutazona, Talniflumat).

Even from early screening and testing of some the chiral aryl alkanoic acids, it was observed that the stereochemistry of the molecule plays an important role in determining the anti-inflamatory activity; that only one of the enantiomers presents such activity. [11]

Nevertheless, with the exception of Naporxen and Flunoxaprofen, marketed as S-enantiomer, all others are used as racemic mixtures.

The profenic structure drugs (derviates of 2-arylpropionic acid), one of the most important anti-inflammatory non-steroidal drugs, discovered in 1961 by Adams, Nicholson and Burrows, are a classical example of the importance of the chirality in expressing the biological effect.

In this thesis, we studied two medicinal substances from the class of non-steroidal anti-inflammatory drug: Ibuprofen and Ketorolac. Both substances have a chiral structure but are marketed as a racemic mixture.

One of the objectives of this thesis is to study the pharmacokinetics of Ibuprofen and Ketorolac, after administration in human volunteers as racemic mixture in different pharmaceutical forms. For Ketorolac it was studied the pharmacokinetics of each enantiomer. The pharmacokinetic analysis of these two drugs imposes to develop the bioanalytical method for quantifying the respective medicinal substance from human plasma. Another objective of this thesis is to study the bioavailability of Ibuprofen and Ketorolac after administration in human as oral solid dosage pharmaceutical forms.

One of the most important properties of a drug is its capacity to release the active substance in such way that it reaches at the action place in a sufficient quantity to obtain the therapeutic effect followed. This property was called physiological bioavailability, biological bioavailability or bioavailability. In other terms, the concept of bioavailability is defined as the speed and the size of absorption of a medicinal substance from pharmaceutical form in the systemic circulation. [12,13]

As it is known that the therapeutic effect of a medicine is correlated with the concentration of the respective medicine in plasma or in blood, the importance of bioavailability in medicinal therapy consists in the fact that the speed and the dimension of drug absorption could influence the pharmacological response. [14]

It was extensively considered that the therapeutic performance is ensured only by the existence of the declared content of medicinal substance in the drug. Further, it was discovered later that the variation in the bioavailability of the drug, correlated with the patients variability is responsible for the therapeutic failure .There are many factors who can influence the bioavailability of the drug, factors that can be correlated with the patient and with the pharmaceutical formulation.

The factors correlated with the pharmaceutical formulation and who can produce high differences in the bioavailability of solid oral dosage form could be related to the medicinal substance (chemical, physical or physic-chemical properties), factors related to the nature, quantity and reactivity of the excipients used or technical factors. [15]

The administration of a medicinal under different pharmaceutical formulations (granules, capsules, tablets, film coated tablets), could, of course, cause variations in the absorption' size and speed. A reproducible bioavailability is related to the absorption' size and speed of the medicinal substances from the pharmaceutical formulation. [16]

The equal bioavailability of two pharmaceutical formulations, evaluated in standard condition, on the same group of healthy volunteers is defining ***bioequivalence.*** For generic products the bioavailability is assessed in comparison with the original product bioavailability, in order to ensure the same biological quality of the both products. If two different pharmaceutical formulations are bioequivalent, they can be considered interchangeable because it is expected that both are responsible for the same therapeutic effect.

The kinetic processes at the administration of the medicinal substance in the body, on a specific way are defined by some specific parameters that can be calculated during pharmacokinetic analysis. The analysis is done based on the plasma concentration of the medicinal substance or its metabolite at a specific time after the drug administration. The parameters calculated can be later used for pharmacokinetic characterization of the medicinal substance, for comparing the quality of some pharmaceutical products or for establishing or individualization of a treatment scheme, based on the specific physiological characteriscs of the body.

In 1992, FDA regulated very clear the discovery of the new chiral drugs, the same was furthered applied in other countries, including European countries, Canada, Australia and Japan.[17] These rules will further encourage the discovery and development of new chiral drugs.

In this context, it becomes very important the development of stereospecific procedures for obtaining chiral molecules with complex structures and also it becomes important the development of efficient techniques for separation of the old / new chiral drugs.

A second objective of the thesis was the study and the establishing of some biocatalytic methods for resolution of the Ibuprofen enantiomers.

B. EXPERIMENTAL PART

B.KETOROLAC PHARMACOKINETICS

B.1.1 MATERIALS AND METHODS

It was realised a clinical study with 22 healthy volunteers having the purpose to study the pharmacokinetics of ketorolac enantiomers after single oral dose administration of ketorolac. It was administrated orally, in fasting condition, a single dose from 2 pharmaceutical formulations: Test, a new experimental formulation, orodispersibile tablets and Reference, the original product, film coated tablets. It was used an enantioselective analytical method for assaying ketorolac from human plasma.

Subjects

22 subjects have participated at the clinical study.

The study protocol was approved by Romanian Agency for Medicine and Medical Devices, Institutional Ethics Committee of "Iuliu Haţieganu" Medicine and Pharmacy University Cluj Napoca.

Before participation at the screening procedures for enrolling in the study, each volunteer has signed and informed consent, after he was previously informed about the study, medicine (dose, therapeutic effects, adverse reactions) and about the study program (drug administration and blood sampling schedule). Only volunteers found healthy based on medical history, medical exam and blood and urine sample analysis results were enrolled in the study. The healthy status of the volunteers was assessed at the end of the study, too.

Study design

The study design was a 2 period crossover study. In each period 10 mg of ketorolac from Test (T) and Reference (R) products were orally administrated, after minimum 10 hours of fasting. The washout period was 7 days.

Blood sampling schedule was: 0, 0.08, 0.17, 0.25, 0.33, 0.5, 0.67, 0.83, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 16, 20, 24 hours. 5 ml of blood were sampled from vein. The bold samples were centrifuged; plasma was separated and stored at –60 °C till analysis.

B.1.2 ANALITYCAL METHPOD AND VALIDATION

Description

The analytical study was done for simultaneous determination of *R*(+)-Ketorolac and *S*(-)-Ketorolac concentration in human plasma, in the clinical study samples, in the presence of internal standard S(+)-Etodolac .

The analytical method was validated as per FDA Guidance for industry referring to bioanalytical method validation [18] and European guide for bioequivalence and bioavailability[19].

The bioanalytical method is described below[20].

Quantitation

An HPLC coupled with mass spectrometry method was used for assaying the concentration of *R*(+)-Ketorolac and *S*(-)-Ketorolac in human plasma with K_3EDTA was used.

Aparatus:

- Agilent 1200 Series binary pump
- Thermostatic autosampler Agilent 1200 Series
- Triple quadrupole mass spectrometer detector API 3200, Aplied Biosystems MDS SCIEX

Working conditions

- Column: Chiral AGP 100 x 4 mm, 5µm, Chrom Technologies
- Mobile phase: ammonium formate buffer (10mM, 4.05): acetone: methanol: (68:9:23, v/v/v) at the flow rate of 0.9 mL/min., temperature: 20˚ C
- Injection volume: 10 µl

Biological samples processing

A pipette was used to aliquot 100 µL of spiked plasma samples into polypropylene tubes. To each tube then added 50 µL of internal standard working solution (2500.00 ng/mL, S(+)-Etodolac in methanol-water, 50:50, v/v) and vortex for 30 s. To each tube 400 µL of 5% formic acid in water is then added, and vortex again for 30 s. The supernatant was loaded on SPE cartridge and centrifuged at 3000 rpm for a minute. Before that the Oasis HLB cartridges were conditioned with 1mL of methanol followed by 1mL of water. After loading of the samples, the cartridge was washed with 0.750 mL of water twice and analytes and IS were eluted with 100% methanol. The extracted samples were evaporated to dryness and reconstituted with 200 µL of mobile phase. Transfer the sample into vials and transferred to autosampler. 10 µL was injected into the LC–MS/MS system for analysis.

c) Samples analysis

Analytes were detected by tandem mass spectrometry using multiple reaction monitoring (MRM) of precursor-product ion transitions with 200 ms dwell time, at m/z 256.2/105.1 for R (+) ketorolac, 256.2/105.1 for S(-) ketorolac and m/z 288.2/172.1 for S(-) Etodolac. The retention times are 3.3, 2.2 and 3.4 for R (+) ketorolac, S(-) ketorolac and S(-) Etodolac respectively

The ions mass detected:

R(+)-Ketorolac: *m/z* 256.20 (parent) and 105.10 (daughter)
S(-)-Ketorolac: *m/z* 256.20 (parent) and 105.10 (daughter)
S(+)-Etodolac: *m/z* 288.20 (parent) and 172.10 (daughter**)**

No interferences were found in blank plasma samples at the retention time of analytes and internal standard due to very specific detection

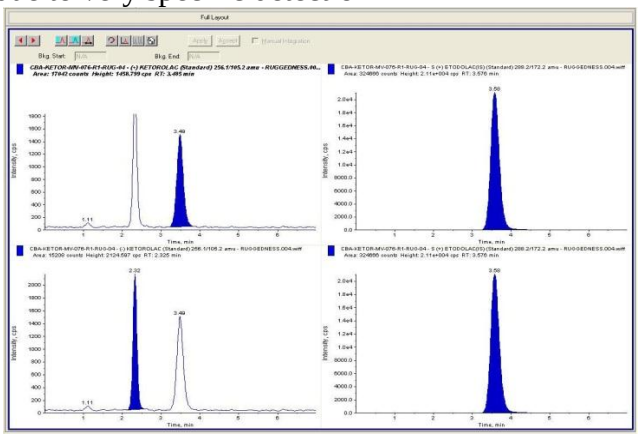
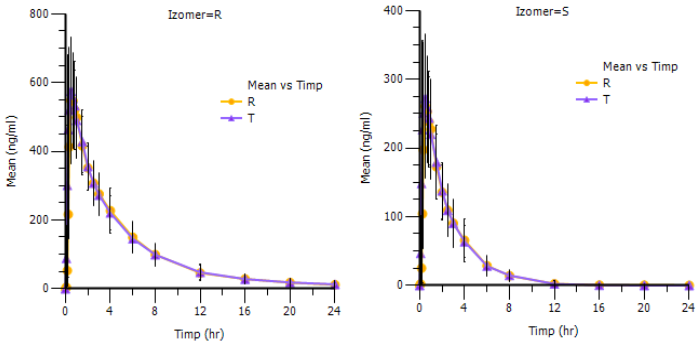


Fig B.1.2. Chromatograms of R(+)-Ketorolac, S(-)-Ketorolac and internal standard S(+)-Etodolac in plasma at the quantification limit concentration.

B.1.3 PLASMA CONCENTRATION OF KETOROLAC

In Fig B.1.7 are presented the average values of plasmatic concentration of *R*- and *S*-ketorolac after pharmaceutical forms administration to the healthy volunteers in the study.



B.1.3.1 NONCOMPARTMENTAL PHARMACOKINETIC ANALYSIS

For non-compartmental pharmacokinetic analysis it was used the program Kinetica 4.2. The following pharmacokinetic parameters were calculated: maximum measured concentration (C_{max}), time of the maximum measured concentration (T_{max}), the area under the concentration versus time curve from time zero to the last measurable concentration (AUC_{last}), the area under the concentration versus time curve from time zero to infinity (AUC_{inf}) and the percentage of extrapolated area under the plasma concentration versus time curve from the last measurable concentration to infinity (AUC_%), the apparent first-order terminal elimination rate constant (Lambda_z), the apparent first-order terminal elimination half-life ($t_{½}$), mean residence time (MRT), the apparent clearance (Cl_F), the apparent distribution volume (Vz_F).

A box-plot comparison for few pharmacokinetic parameters for *R*(+)- and *S*(-)-ketorolac resulted from the study is presented in Fig B.1.8-B.1.9. In this graphical representation it is visually compared the average values of pharmacokinetic parameters of these 2 enantiomers, as per the product dosed.

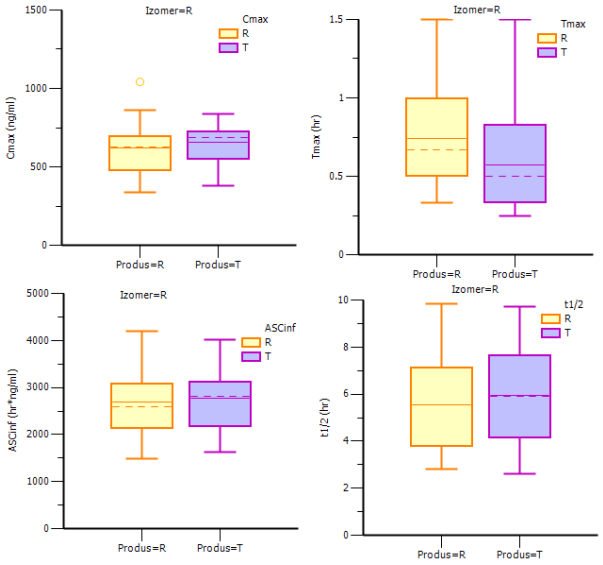


Fig B.1.3 Average values of PK parameters of *R*-ketorolac for Test and Reference products

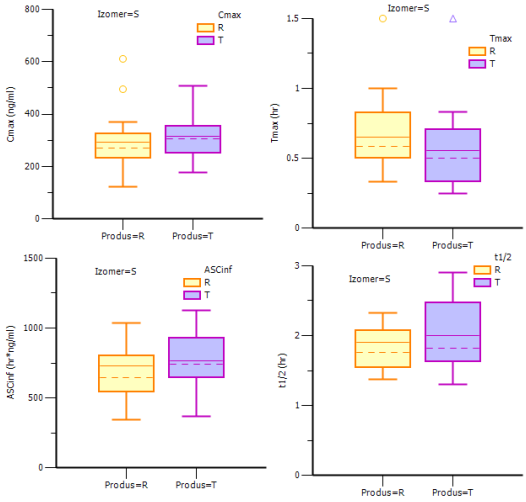


Fig B.1.4 Average values of PK parameters of *S*-ketorolac for Test and Reference products

Pharmacokinetic parameters analysis indicates that these 2 enantiomers are very different after dosing as racemic.

The *R*(+)enantiomer presents a C_{max} double comparing with the *S*(-) enantiomer, very well correlated with the mean residence time in the body MRT of approx. 6 hours for *R*(+) enantiomer.

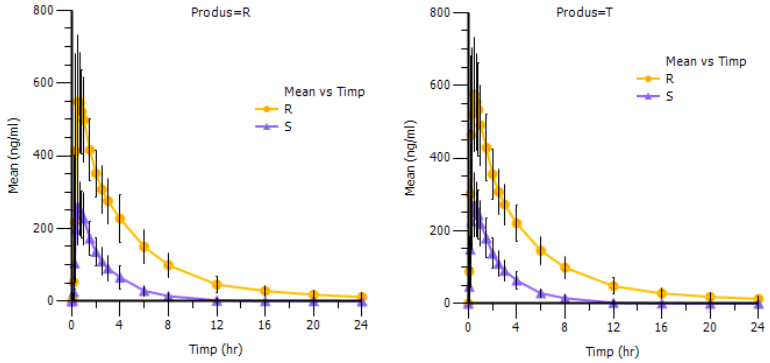
The S(-) enantiomer, responsible of the anti-inflammatory activity of ketorolac is eliminated almost 3 times quicker than R(+) enantiomer.

Enantiospecific pathway is reproducible from Test product to Reference product, the data obtained from these 2 very different pharmaceutical forms (film coated tablets and orodispersabile tablets) being very consistent.

Comparative analysis of pharmacokinetics of R & S ketorolac enatiomers

It was performed the comparative statistical analysis for the individual values of PK parameters of R(-)- and *S*(+)-ketorolac calculated before.

The average concentrations of ketorolac enantiomers, for each product studied, are presented in Fig. B.1.5.



The pharmacokinetics parameters of the enantiomers are significantly different from statistical point of view. The biggest difference is observed for area under the curve AUC and for C_{max}, which are very well correlated with elimination rate, different for these two enantiomers.

One exception is observed for T_{max}, the difference between the enantiomers is not statistical significant. These results are confirming once again the existence of a different pharmacokinetic for these two enantiomers.

B.2 IBUPROFEN BIOAVAILABILITY

B.2.1 MATERIALS AND METHODS

There were realised 3 clinical studies, in Fasting and Fed condition, for studying the pharmacokinetic of Ibuprofen after single oral dose administration in healthy volunteers. (Table B.2.1).

TableB.2.1. The main characteristics of the clinical studies realised for studying the Ibuprofen's pharmacokinetics.

Study (cod)	Study type (Food)	Pharmaceutical formulations	No of subjects	Study design
STD1	Fasting	Test (T), Reference (R)	29	Crossover
STD1	Fed	Test (T), Reference (R)	31	Crossover
STD2	Fasting	Test1 (T1), Test2(T2), Reference (R)	9	Crossover
STD2	Fed	Test1(T1), Test2(T2), Reference (R)	9	Crossover
STD3	Fasting	Test (T) , Reference (R)	60	Crossover
STD3	Fed	Test (T), Reference (R)	31	Crossover

Subjects

In these three studies, in the both versions, fed and fasting, 9 to 60 volunteers have participated. (Table B.2.1).

The clinical studies protocols were approved by Romanian Agency for Medicine and Medical Devices, Institutional Ethics Committee of "Iuliu Haţieganu" Medicine and Pharmacy University Cluj Napoca.

Before participation at the screening procedures for enrolling in the study, each volunteer has signed an informed consent, after he was previously informed about the study medicine (dose, therapeutic effects, adverse reactions) and about the study program (drug administration and blood sampling schedule). Only volunteers found healthy based on medical history, medical exam and blood and urine sample analysis results were enrolled in the study. The healthy status of the volunteers was assessed at the end of the study, too.

Study design

Each study was conducted in 2 periods (STD 1 and 3) or 3 periods (STD 2), being a crossover study, in each period it was alternatively, orally administered a dose of 400 mg ibuprofen (STD 1) or 200 mg ibuprofen (STD 2 and 3) to study subjects. There were administered experimental formulations (treatment or pharmaceutical product noted with T, T1 and T2) and reference formulations (treatment or pharmaceutical product noted with R). The washout period was for a week.

The blood sampling schedule was as follows:

STD1, Fasting: 0, 0.167, 0.33, 0.5, 0.67, 1, 1.25, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, 12 hours

STD1, Fed: 0, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.33, 2.67, 3, 3.5, 4, 5, 6, 8, 10, 12 hours

STD2, Fasting: 0, 0.167, 0.25, 0.33, 0.5, 0.667, 0.833, 1, 1.25, 1.5, 2, 2.5, 3, 5, 7, 9, 12 hours

STD2, Fed: 0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.25, 2.5, 2.75, 3, 3.5, 4, 5, 6, 8, 12, 14, 18 hours

STD3, Fasting: 0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.5, 3, 4, 5, 6, 8, 10, 12 hours

STD3, Fed: 0, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.25, 2.5, 3, 4, 5, 6, 8, 10, 12 hours

Blood samples were collected (5 ml, through a vein puncture) after dosing.

After centrifugation of blood samples, plasma was separated and stored at -60 ° C until analysis.

B.2.2 ANALYTHICAL METHOD AND VALIDATION

Description

The analytical study was performed to measure plasma concentrations of ibuprofen in biological samples from clinical studies.

This study was conducted in accordance with clinical and analytical protocol approved by the competent authorities and with Good Clinical Practice guidelines, respectively the principles of Good Laboratory Practice. The analytical method was validated as per European Guide for bioequivalence and bioavailability 1498/2010Rev 1[7] and FDA Guidance for industry referring to the bioanalytical method validation.[6]

It was used a simple, rapid and sensitive LC / MS / MS method for quantification of ibuprofen in plasma.[21i]

Quantitation

An HPLC coupled with mass spectrometry method was used for assaying the concentration of ibuprofen in human plasma[22i].

Aparatus:

- Agilent 1100 Series binary pump
- Thermostatic autosampler Agilent 1100 Series
- ion trap mass spectrometer detector Ion Trap VL, Agilent

Working conditions:

- Column: Zorbax RX C18 3.0 x 100mm, 3µ (Agilent Technologies)
- methanol : ammonium acetate 10 mM in water 60:40, v/v
- Flow rate: 0.3 mL/min, temperature: 25˚ C
- Injection volume: 30 µl

Biological samples processing

To 1000µl plasma add 100 µl internal standard (sodium diclofenac), vortex-mix for 5 seconds at 1500 rpm, add 100 µl phosphoric acid 1.4N, vortex 5 seconds at 1500 rpm. To the mixture, add 5 ml mixture diethyl ether: hexane = 80: 20. Shake the samples for 25 minutes at 1000 rpm, centrifuge and then transfer 4 ml of organic layer to another polypropylene tube. Evaporate to dryness at 50 °C, under a stream of nitrogen. The residue is reconstituted with 200 µl mobile phase, vortex for 1 minute at 2400 rpm, centrifuged and injected into HPLC system. In the electric field created by the electrospray ionization source, the molecule of ibuprofen readily loose a proton, a negative ion being generated, with m/z 205. Thus, the detection of ibuprofen was carried out in multiple reactions monitoring (MRM) mode, by fragmentation of the parent ion with the m/z 205. The fragmentation of the parent ion is induced by collision of the ion with helium in the ion trap and stable product ions with m/z 159 and m/z 161 are observed in the mass spectra. Finally, the sum of abundance of ions with m/z 159 and m/z 161 was monitored and quantitatively correlated with IBU concentration. Sodium diclofenac was used as internal standard, the MS transition monitored in its case being m/z 250 > m/z 293, in positive mode The retention time of IBU was 4.7 min and, due detection specificity, no significant interference was observed at the retention time in plasma blank samples

B.2.3 PLASMA CONCENTRATIONS OF IBUPROFEN

All pharmacokinetic and statistical calculations presented in this chapter are exclusively based on the values of plasma ibuprofen concentrations.

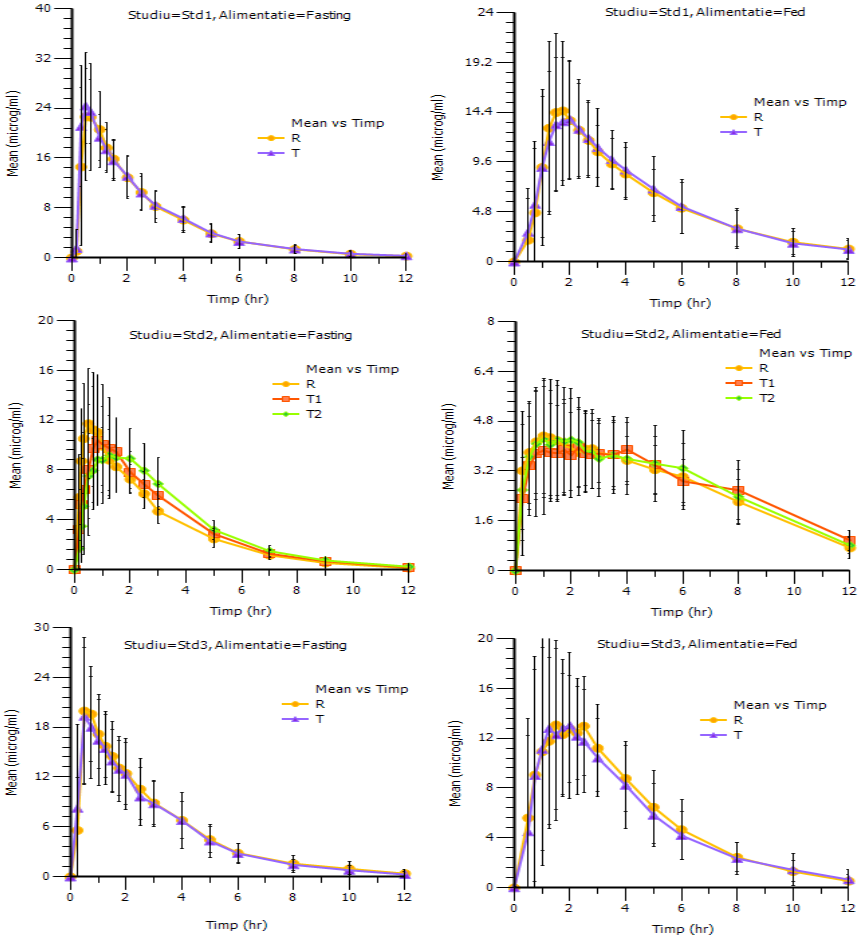


Fig B.2.4 Average plasma concentration of ibuprofen after single oral dose administration to healthy volunteers

B.2.3.1 NON-COMPARTMENTAL PHARMACOKINETIC ALALYSIS

For non-compartmental pharmacokinetic analysis it was used the program Kinetica 4.2. The following pharmacokinetic parameters were calculated: maximum measured concentration (C_{max}), time of the maximum measured concentration (T_{max}), the area under the concentration versus time curve from time zero to the last measurable concentration (AUC_{last}), the area under the concentration versus time curve from time zero to infinity (AUC_{inf}) and the percentage of extrapolated area under the plasma concentration versus time curve from the last measurable concentration to infinity (AUC_%), the apparent first-order terminal elimination rate constant (Lambda_z), the apparent first-order terminal elimination half-life ($t_{½}$), mean residence time (MRT), the apparent clearance (Cl_F), the apparent distribution volume (Vz_F).

The analysis of pharmacokinetic parameters obtained in ***Study 1*** shows that ibuprofen administered in fasting conditions has a C_{max} of approximately 26 µg/mL for both formulations administered, at a T_{max} of approximately 0.6 hours. The total area under the concentration versus time curve was of 65-67 µg/mL*hr for both formulations, and the mean residence time in organism was approximately 2.9 hours. In case of ibuprofen administered in fed conditions the pharmacokinetic parameters are significantly modified. Thus, the maximum measured concentration decreases to 17 mg/mL, the time of the maximum measured concentration is increased at 2 hours. Also, the total area under the concentration versus time curve is increased at 74 µg/mL*hr and the mean residence time became approximately 5 hours. Similar observations can be made for the other two studies.

B.2.3.2 COMPARATIVE BIOAVAILABILITY. BIOECHIVALENCE OF IBUPROFEN

Firstly, it was performed a comparative statistical analysis of pharmacokinetic parameters of ibuprofen administered in these three studies, fed or fasting, variability sources considered were the type of formulations and the subjects (2-way ANOVA analysis). The results of the statistical analysis performed are presented in tables B2.23-B.2.25a-c. It was considered significant from statistical point of view if p value is <0.05.

Analysing the results of statistical tests in the tables presented before, it can be observed that a common feature is that because of inter-individual variability (inter-subject), for the most pharmacokinetic parameters, there are statistically significant differences within the group. In some cases, however, no statistically significant differences were found between subjects for parameters such as T_{max}, MRT and $t_{1/2}$ parameters with lower inter-individual variability.

Regarding statistical differences of pharmacokinetic parameters of ibuprofen dependent on the pharmaceutical product (test or reference) administered in each study, it is observed that in most cases, they are not statistically significant (STD1/Fasting and Fed, STD3/Fed). In some cases statistically significant differences were detected for parameters such as C_{max} (STD2/Fasting) or AUC_{inf} (STD2/Fasting and Fed STD3/Fasting). These differences are due to the biopharmaceutical properties of the dosage form used.

For the pharmacokinetic parameters C_{max}, AUC_{last} and AUC_{inf}, a bioequivalence test was performed by calculating 90% confidence interval of the mean log ratio values (ANOVA) (Table B.2.26). In accordance with national and international [6,7] requirements, products are considered bioequivalent if the calculated confidence interval value is between [0.8-1.25]. The values obtained, regardless the study type and the fed/fasting condition when taking ibuprofen, fall within the bioequivalence interval of 80-125%, so all test products are bioequivalent to the reference products.

For the T_{max} parameter it was calculated the difference in mean non-logarithmic

values of test product and reference product and the Friedman test was applied to detect significant differences. In each case examined, the differences found for T_{max} parameter were not statistically significant

B.2.3.3 FOOD EFFECT OVER THE IBUPROFEN PHARMACOKINETICS

In chapter B2.3.2 it was shown that the formulation administered (test or reference) had no significant influence on the pharmacokinetics of ibuprofen.

Therefore, for studying the effect of food on the pharmacokinetics of ibuprofen, there were calculated mean plasma concentrations of ibuprofen for each clinical study, depending on diet, but without taking into account the type of formulation administered (Figure B.2.9)

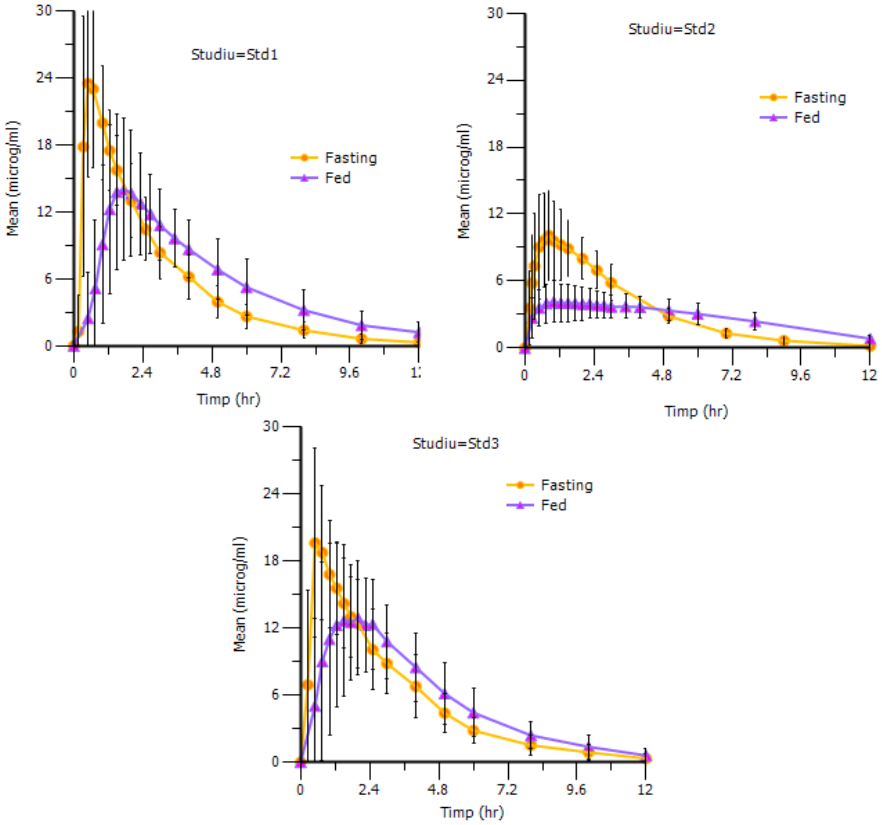


Fig B.2.9. Influence of food on plasmatic profiles of ibuprofen

For all three clinical studies, there is a strong influence of food on plasma concentrations of ibuprofen, which will later reflect differences in its pharmacokinetic parameters.

A statistical analysis was performed on the pharmacokinetic parameters of ibuprofen for each study (one-way ANOVA analysis), the source of variability was food. The results obtained, for each study respectively for the pharmaceutical product administered, are shown in Tables B.2.30a-c

Analysis of statistical results presented in previous tables shows that the pharmacokinetic parameters correlated with the rate of absorbtion, are significantly modified under the influence of food (C_{max}, T_{max}). Also, mean residence time MRT which indirectly depends on plasma concentrations over time is statistically significantly influenced by food.

Because food decreases absorption rate, this could become in some cases lower than the elimination rate, and the terminal slope of the graph ln (concentration) versus time will reflect the absorption constant and not the elimination constant (flip-flop phenomenon). For

this reason, in lambda_z values (and thus $t_{1/2}$ values) there were found statistically significant values depending on food. However, the parameters that are less dependent on the absorption rate and more on the extent of absorption (area under the curve - AUC_{last}, AUC_{inf}) are in general not significantly affected by food, which proves that the mass of ibuprofen absorbed, and the one that reaches the systemic circulation is relatively equal between the absence/presence of food.

B.2.4 COMPARTMENTAL PHARMACOKINETIC ALALYSIS

Unlike non-compartmental pharmacokinetic analysis that is independent of the model, compartmental analysis assumes preliminary considerations on the kinetic processes involving the drug substance. These processes can be absorption, distribution, metabolic or renal elimination. In compartmental pharmacokinetic analysis there are created pharmacokinetic models with a series of mathematical equations, each representing a specific kinetic process.

Using plasma concentrations of ibuprofen obtained from the pharmacokinetic experiment described in Chapter B.2.5, compartmental pharmacokinetic analysis was performed for these two substances.

For compartmental pharmacokinetic analysis was used the computer program WinNonlin (Pharsight).

Compartmental pharmacokinetic analysis method

The compartmental pharmacokinetic analysis purpose is, firstly, the identification of the pharmacokinetic model that best describes the plasma concentrations of the substance obtained from clinical and analytical study. Then, using the optimal pharmacokinetic model there can be calculated the specific pharmacokinetic parameters of the model and that describe the kinetic processes of the drug substance in the body.

The selection of the model that best describes the pharmacokinetics of the substance was achieved using a series of statistical tests that expresses quantitatively the accuracy suitability. In this study the primary criteria used for discriminating competing proposed models was index Akaike (noted AIC). There were analyzed using 6 pharmacokinetic models, mean plasma profiles of ibuprofen in each study/type of diet, finally there were 6 sets of data.

Monocompartmental pharmacokinetic model

There were four mono-compartmental models proposed for pharmacokinetic analysis of ibuprofen, the differences between them consist in the existence or not of a latency time until the start of the absorption (more specific, until the appearance of the substance into the systemic circulation) respectively considerations regarding the relative values of the constants of absorption and elimination processes ($k01=k10$ or $k01\neq k10$).

The first model (M1) considers that the absorption takes place after a 1st order kinetics with no latency time and $k01\neq k10$.

The second model 2 (M2) also considers that the absorption takes place after a 1st order kinetics, but it does not start immediately after administration, but after a latency time (t_{lag}), also $k01\neq k10$.

The third and fourth models (M3 and M4) are similar models with M1 and M2 characterized above, except that $k01= k10$.

The fifth model (M5) considers the absorption kinetics of 1st order, $k01\neq k10$, bi-compartmental distribution without absorption latency time. The sixth model (M6) is similar to model 5, but assumes an absorption latency time.

Bicompartmental pharmacokinetic model

Compared with the monocompartimentat model described above, this model considers that there may be equilibrium distribution of ibuprofen between the central compartment and a peripheral compartment. As in the previous case, there will be tested two pharmacokinetic models, the difference among them is that there is a latency time at M6 model from M5 model (where absorption starts immediately).

Choosing the best simple pharmacokinetic model

The pharmacokinetic model best correlated with experimental data is characterized by a minimum Akaike index. To identify it, there were compared the mean values of Akaike of the 6 models discussed above (Fig. B.2.17).

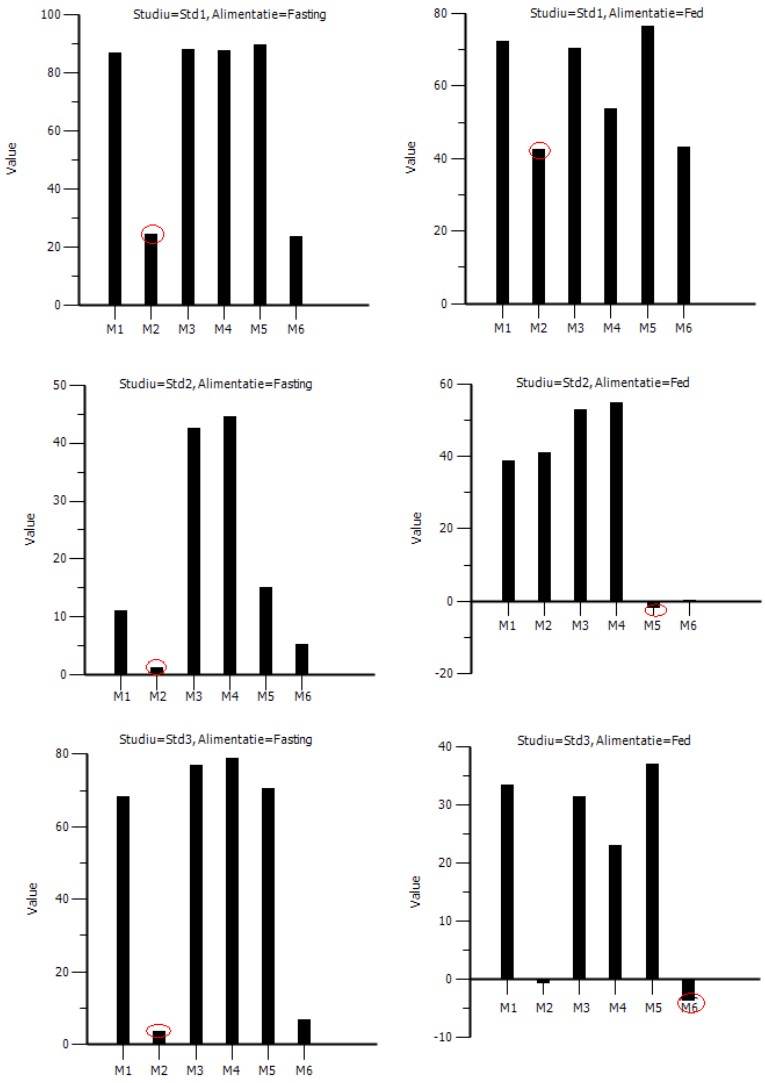


Fig B.2.17 Akaike values for pharmacokinetic models tested compared to the analyzed data sets

As it can be observed analyzing Fig.B.2.17, with two exceptions, the pharmacokinetic model M2 best fits ibuprofen plasma profiles under different formulations (studies 1-3), with or without food, followed closely by model M6. The two exceptions are the data from study

2/Fed, for which the optimal model is M5, respectively 3/Fed study, for which the optimal model is M6

Regarding the data from 2/Fed study, although apparently the best model is M5, it should be borne in mind that the analysis shows that M5 model is the best studied model without being a guarantee that there is not a model more appropriate. An analysis of data matching study 2/Fed with M5 model (Figure 15, profile 4) shows that the terminal plasma profile is tough not well suited to model M5. Moreover, a graphical representation of the predicted concentration versus the determined concentration, which for an ideal fit is a line equal to the identity, shows a systematic deviation from the identity line, particularly at low concentrations. In other words, even this model does not describe satisfactorily the pharmacokinetics of ibuprofen administered STD 2/Fed. Probably the effect of food on the absorption rate of ibuprofen is complex and plasma data can not be analyzed using classical pharmacokinetic models.

Regarding the data from STD 3/Fed that are best suited with model M6, it should be borne in mind that this is very similar to M2 model only that it assumes also the distribution processes. Although M6 model is indicated as the best model by Akaike value, it must have a capacity of pharmacokinetic constants calculation with adequate accuracy. A comparative analysis of coefficients of variation of the standard error of the mean, which characterizes the precision of the determination of a pharmacokinetic parameter value, for M2 and M6 models applied to data from STD 3/Fed, is presented in Table. B.2.31

Analyzing the data presented in Table B.2.31, it is observed that although M6 model fits better the data from STD 3/Fed still has a reduced capacity to estimate *accurately* the calculated parameters, the percentage error reaching even to 98% for K12 value. Instead, the model M2 has a much better capacity to estimate accurately the parameters, the maximum percentage error being 7.48%. For this reason, it will be considered a representative model for STD 3/Fed also the model M2 and not M6.

After comprehensive analysis of compartmental pharmacokinetic analysis results for 5 of the 6 combination study/type of diet showed that optimal pharmacokinetic model is M2, while for STD 2/Fed the optimal pharmacokinetic model was not identified due to the complexity of the absorption under the influence of food.

In conclusion, it was shown that ibuprofen pharmacokinetics is best described by the mono-compartmental pharmacokinetic model, 1st order absorption kinetics with latency time and using this model there were determined individual and mean pharmacokinetic parameters of ibuprofen.

B3. BIOCATALYSIS

B3.1.BIOCATALYSIS WITH *SACCHAROMYCES CEREVISIAE*

Among microorganisms, baker's yeast is very often used as a general biocatalyst. The asymmetric reduction of carbonyl group is one of the most important, fundamental and practical method for obtaining chiral alcohols, which can be further transformed in a large variety of organic compounds. The asymmetric reduction can be realized by chemical or biochemical procedures. As result of substrate-enzyme interaction, a high regio-, chemo- and enantioselectivity are obtained. Dehydrogenases and reductases, classified as E.C. 1.1.1. are enzymes which are involved in the carbonyl group reduction. Their natural substrate is the ethanol, glycerol, lactate etc, but they can reduce also various unnatural ketones in a stereoselective manner, according with the Prelog's rule.

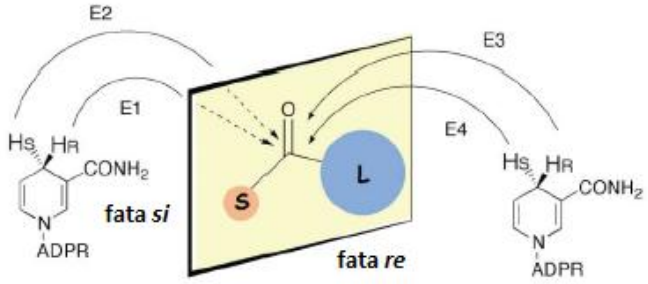


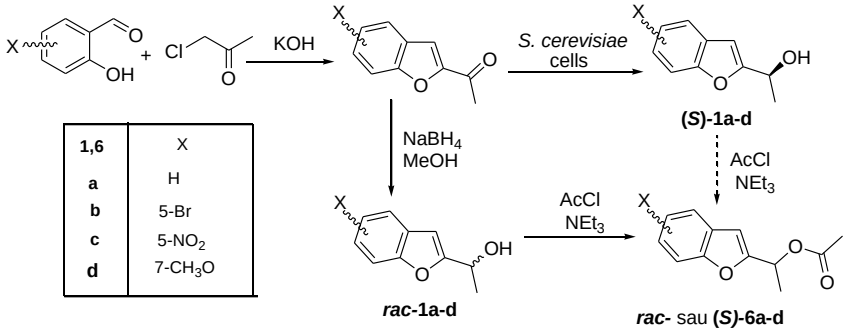
Fig. B3.3. The stereochemistry of hydride transfer on the carbonyl group, according to the Prelog's rule (L-small group, L-large group)

B.3.1.1 Enantioselective bioreduction of acetyl-benzofurans and their α-subtituted derivatives with S. cerevisia cells for obtaining chiral heteroaromatic ethanols

(Benzofuran-2-yl) carbinols exhibit various biological activities. Such derivatives were Investigated as antibacterial or antifungal agents. Moreover, optically active 2-(2-*tert*-butyl-amino-1-hydroxyethyl) benzofurans were investigated as β-blockers. 2-Substituted benzofurans can inhibit the HIV-1 reverse transcriptase or act as antiaging compounds. Baker's yeast reduction of hydroxymethyl ketones and acetoxymethyl ketones proved to be useful for production of opposite enantiomeric forms of 1,2-diols. Ketones with a relatively small and hydrophilic hydroxymethyl group were all reduced from the same face, whereas the acetoxymethyl ketones were reduced with the opposite enantiotopic preference.

B.3.1.1.A Synthesis of some chiral ethanols by enantioselective bioreduction of 2-acetylbenzofuran derivatives

2-Acetylbenzofurans were synthetized from the potassium salt of the corresponding salycylaldehyde and monochloroacetone in benzene (Scheme B3.1).



Scheme B3.1. Synthesis and reduction of ketones with *S. cerevisiae* cells

First the reduction of 1-(benzofuran-2-yl)etanone was studied under fermenting and non-fermenting conditions. In the fermenting system, the reduction was faster but less selective (80% yield, 2 days, 20% ee). Therefore, the non-fermenting system (60% yield, 7 days, 55% ee) was applied for reduction of the further (benzofuran-2-yl)ethanones as well (Table B3.1).

Table B3.1. Baker's yeast reduction of 1-(benzofuran-2-yl)ethanones in nonfermenting system after 7 days

Product	Yield (%)	$[\alpha]_D^*$	ee (%)
(*S*)-**1a**	60	-9.1	55
(*S*)-**1b**	74	-9.4	65
(*S*)-**1c**	61	-16.8	88
(*S*)-**1d**	66	-10.2	68

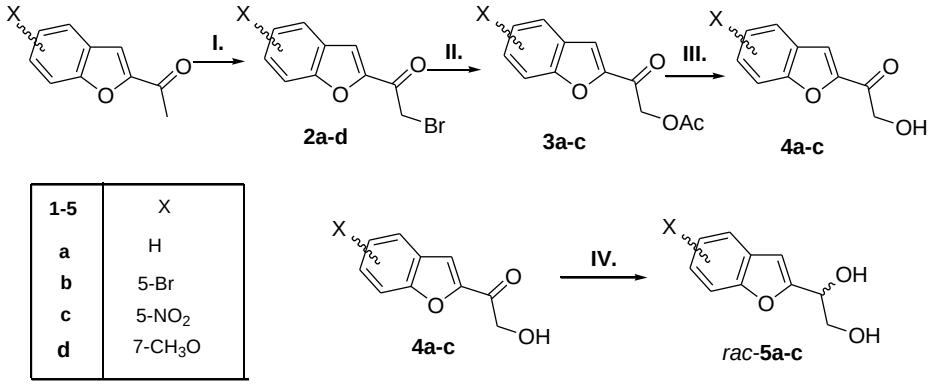
*at 20 °C, in chloroform

The enantiomeric composition of the products were precisely determined by GC and HPLC on chiral columns. Comparison of the specific rotation of our sample to the rotation value of (1*R*)-1-(benzofuran-2-yl)ethanol established its (*S*)-configuration. [24,23] This (*S*)-configuration was also expected on the basis of the Prelog's rule. The enantiomeric composition of the alcohols (*S*)-**1a**–**d** was determined after conversion into their acetates (*S*)-**6a**–**d** by GC and HPLC.

B.3.1.1.B. Biotransformation of α-subtituted ketones with S. cerevisiae cells for obtaining chiral ethandiols

To investigate the influence of different substituents upon *Saccharomyces cerevisiae* mediated biotransformtion,[25,24] the α-derivatisation of 2-acetylbenzofurans was proposed, following the reaction sequence presented in Scheme B3.2. A new one pot procedure was latterly developed.[26,25]

A three-step chemoenzymatic sequence for the indirect conversion of methyl ketones into hydroxymethyl ketones **4a–c** *via* acetates **3a–c** obtained by 18-crown-6 catalysed substitution of the corresponding bromomethyl ketones **2a–c** with NaOAc, followed, as the final step, by a mild and efficient lipase-catalysed ethanolysis. The methoxy derivative proved to be unstable.



Scheme B3.2. Synthesis of α-substituted ketones **2,3,4a-c** and of racemic ethane diols *rac*-**5a-c**

I. Br_2,/CH_2Cl_2; II. $CH_3COO^-Na^+$, 18C6/1,4-dioxane; III. Novozyme 435/EtOH; IV. $NaBH_4$/MeOH

The enantiomeric composition of the diols (*R*)-**5a-c** or (*S*)-**5a-c** was determined by HPLC on (*S*,*S*)-Whelk-O1 (Figure B3.4).

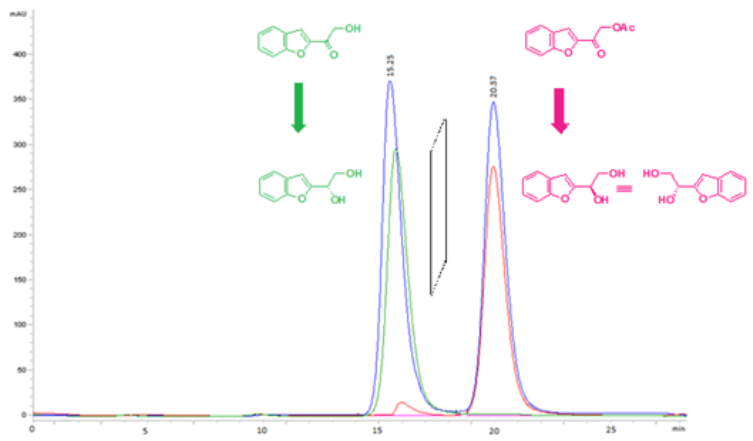


Fig. B3.4. The chromatogram of the: racemic ethandiol *rac*-**5a** (blue line) in comparison with those obtained by biotransformation with *S. cerevisiae* cells of α-hydroxy-ketone **3a** ((*S*)-**5a**, green line) and α-acetoxy-ketone **3a** respectively ((*R*)-**5a**, red line)

In Table B3.3 are presented the results obtained in nonfermenting system when the prochiral ketones **3,4a-c** were transformed with *S. cerevisiae* cells.

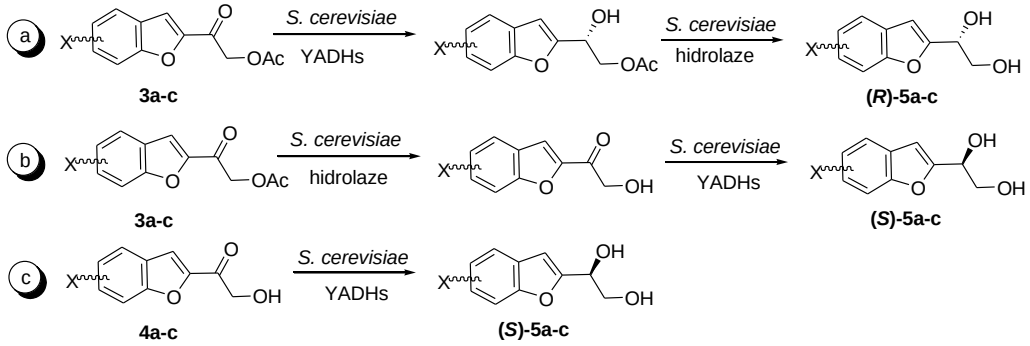
Table B3.2. Biotransformation with *S. cerevisiae* cells in nonfermenting system of prochiral ketones **3,4a-c**

Ketone	Product	Time (h)	Yield (%)	$[\alpha]_D^*$	ee (%)
3a	(*R*)-**5a**	72	75	+24.1	84
3b	(*R*)-**5b**	72	82	+21.7	91
3c	(*R*)-**5c**	72	80	+19.0	91
4a	(*S*)-**5a**	24	60	-25.3	87
4b	(*S*)-**5b**	24	74	-22.1	92
4c	(*S*)-**5c**	24	61	-19.4	93

*at 20 °C, in chloroform

It was found that the various additives significantly influenced the selectivity of the reduction (Table B.3.3.). The moderate 42% ee enantiomeric excess observed with the fermenting system (entry 1) has been increased to 80% ee when the fermentation was performed in a hexane water two-phase system (entry 4) or to 82% ee by adding L-cysteine (entry 5). The highest ee of the produced diol [(*S*)-**6a**, 87% ee); however, was achieved when the fermentation was carried out under non-fermenting conditions (entry 6). Because non-fermenting conditions proved to be the most selective for reduction of the test hydroxymethyl ketone **3a**—similarly as for the simple ketones, bioreduction of the other hydroxymethyl ketones **3a**–**c** and acetoxymethyl ketones **4a**–**c** were also performed in this way (Table B3.3).

Interestingly, from the reduction of the acetoxymethyl ketones **3a**–**c** alcohols (−)-**5a**–**c** were exclusively isolated indicating that the benzofuran-2-yl derivatives are good substrates of the hydrolases also present in the baker's yeast cells. According to our expectations, these alcohols (−)-**5a**–**c** had the opposite sign of specific rotation than those (+)-**5a**–**c** obtained from the reduction of the hydroxymethyl ketones **4a**–**c**. The presence of a substituent at position 5 increased the selectivity of the reduction (in the acetoxymethyl series, **3a**–**c**, from 84 to 91% ee; or in the hydroxymethyl series, **4a**–**c**, from 87 to 92 and 93% ee).



*Scheme B3.3***.** Possible reaction during the biotransformation of **3,4a-c** with *S. cerevisiae* cells

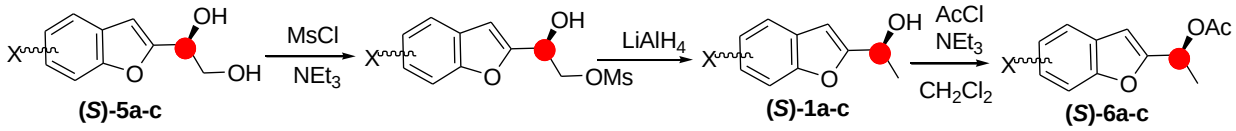
Table B3.3. Influence of the conditions on the selectivity of asymmetric reduction of 2-hydroxy-1-(benzofuran-2-yl)ethanone **4a** in fermenting system after 24 ore

No.	Additive (amount)	Yield (%)	Absolute configuration[a] 5a	ee^b_{5a} (%)
1	-	75	*S*	42
2	Cloroacetat de etil (0.5%)	36	*S*	54
3	Alcool alilic (0.5%)	46	*S*	61

4	Hexan (1:1, v:v)	56	*S*	80
5	L-cisteina (0.5%)	80	*S*	82
6	[c]	60	*S*	87

[a] Determined by chemical derivatization; [b] Determined by HPLC; [c] Non-fermenting system (without saccharose).

Because the absolute configurations of the produced diols were not known, the diols (−)-**5a**–**c** prepared by whole-cell biotransformation with *S. cerevisiae* from hydroxymethyl ketones **4a**–**c** were converted to (1*S*)-1-(benzofuran-2-yl)ethanols (*S*)-**1a**–**c** (Fig. 3). Thus, from the (−)-diols (−)-**5a**–**c** (1*S*)-ethanols (*S*)-**1a**–**c** were obtained *via* selective mesylation and $LiAlH_4$ reduction of the resulting mesylates (Scheme B3.4), proving the (*S*)-configuration of diols (−)-**5a**–**c**. Moreover, the 1-(benzofuran-2-yl) ethanols (*S*)-**5a**–**c** were converted into their acetates (*S*)-**6a**–**c** for determination of their enantiomeric composition by GC on chiral phase.



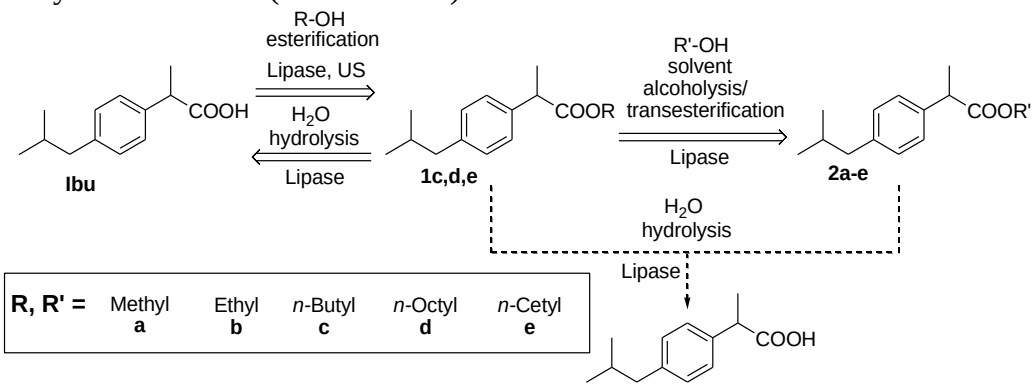
Scheme B3.4. Chemical transformation of 1-(benzofuran-2-yl) ethane-1, 2-diols **5a**–**c** into 1-(benzofuran-2-yl) ethanols **1a**–**c** and their acetates **6a**–**c**.

B.3.2. LIPASE MEDIATED KINETIC RESOLUTION OF RACEMATES

Ibuprofen is one of the most important members of NSAIDs that belongs to the family of propanoic acid. Their anti-inflammatory activity (by cyclooxygenase inhibition) resides primarily in the *S* enantiomer which is more water soluble, promoting more rapid dissolution.[27,26] The undesired (*R*)-profens might bring some health problems, *e.g.* accumulation in fatty tissues, with unknown effects.[28,27]

Biocatalysis offers a green alternative for the resolution of racemic profens using usual kinetic resolution processes. Large series of lipases and esterases have been shown to be highly enantioselective towards several profens. Not only hydrolytic approaches performed in aqueous media, but also alcoholysis and aminolysis reactions in non-conventional media were reported in the last two decades.[29,28]

A systematic study for the enzymatic kinetic resolution of racemic ibuprofen esters with long chain alcohols, which are most closed to the lipase natural substrates, with some commercial available lipases was performed. The effect of alkyl chain length of the alcohol on the performance of all possible processes: esterification, alcoholysis (trans-esterification) and hydrolysis was studied (Scheme B3.5).



B3.5. Possible biocatalytic processes on ibuprofen derivatives in presence of lipases

The presences of lipases as catalyst make all studied possible routes (esterification, alcoholysis/transesterification and hydrolysis) stereoselective. Depend on the enzyme stereoselectivity, *R* or *S* enantiomers of the reaction counter parts could be synthesized. Due to the residual water content of the used solvent and/or enzyme, the alcoholysis was generally concurred by secondary hydrolytic reactions.

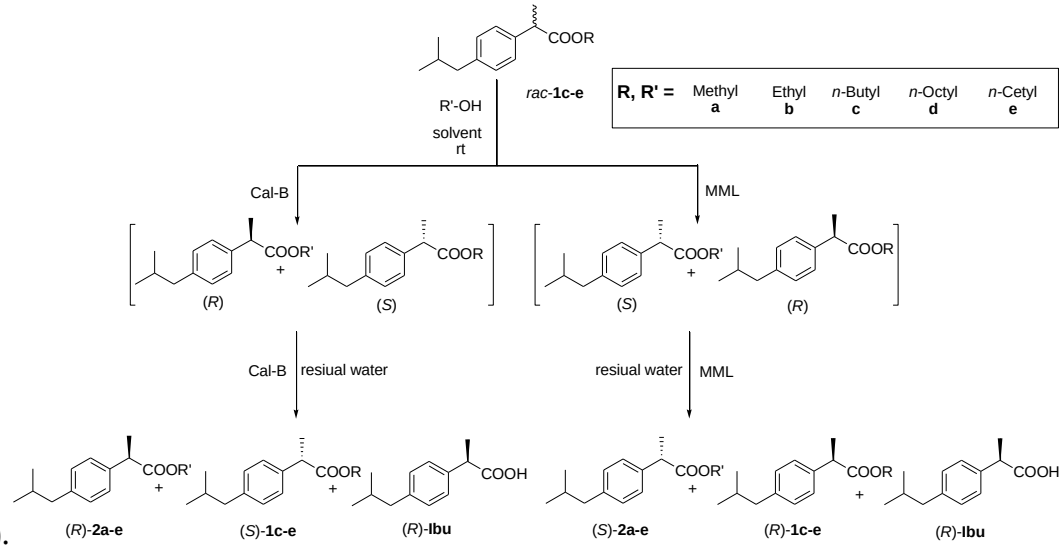
First, the lipase-catalysed esterification of racemic ibuprofen (**Ibu**) was tested under various conditions (Scheme B3.5, route a). Commercial lipases, such as *Candida antarctica* lipases A and B (Cal-A and Cal-B), lipase prom *Candida rugosa* (CRL, AYS Amano) and *Pseudomonas cepaciae* (PS-C II), lipase AK from *Pseudomonas fluorescens*, pancreatic porcine lipase (PPL) and *Mucor miehei* lipase (MML) were screened for the esterification of ibuprofen with several alcohols at room temperature under ultrasound irradiation and/or in batch process. Only the *R*-selective Cal-B was satisfactory as biocatalyst for esterification, leading to the *S* enantiomer of the acid. The presence of ultrasound media increase the reaction rate (c >13% after 1 h in all cases), but leads to lower selectivity (E= 1-5). Using different alcohols as nucleophile (*n*-butanol, *iso*-butanol, *n*-pentanol, *n*-octanol, *n*-hexadecanol) was found that the enzymatic activity in neat alcohol decreased proportionally with the carbon chain length. Best results were obtained with immobilized Cal-B (available at low price as Novozym 435) and *n*-butanol (c= 12%, E = 5).

It is well known that the polarity of the solvent, which can be described by the log *P* value, influence the enzyme activity. For the enzymatic ester synthesis solvents having log *P* >4.0 are generally recommended.[30,29] Next the esterification with *n*-butanol (4 eq) and Cal-B (2:1 weight *ratio*) was performed in several recommended solvents (MTBE, *n*-octane, acetonitrile, toluene, THF), which slightly improve the activity of the enzyme. Solvents such as THF or acetonitrile, more hydrophilic, strip the essential water surrounding the enzyme leading to enzyme inactivation (~ 13% conversion after 1 h), while hydrophobic solvents such as *n*-octane and MTBE preserve or enhance the catalytic activity (20 and 14% conversion after 1 h), but not significant improvement of selectivity was observed.

Next the hydrolysis of three bulkier racemic ibuprofen esters (*rac*-**1c-e**) was performed in presence of same lipases in the same organic solvents, but only Cal-B and MML were weekly active; the conversions were low (<15% after 48 h) and the enantioselectivity low to moderate (E= 1-3).

Bäckvall reported recently[31,30] the use of a triple-fold mutant of lipase A from *Candida antarctica* with a 30-fold enhanced activity towards profens, which displayed high enantioselectivity in the hydrolysis of ibuprofen esters with (*R*) - stereo preference, but still with rather low conversions. On the basis of this report and of our previous results for the lipase mediated *O*-acylation with fatty acids[32,31] we decided to study the alcoholysis (trans-

esterification) of the racemic bulkier ibuprofen esters with various aliphatic alcohols (Scheme B3.6).



Scheme B3.6. The opposite enantioselectivity of Cal-B and MML in the alcoholysis of racemic ester of Ibuprofen

First an extensive screening for the alcoholysis of the racemic ibuprofen octyl ester (*rac*-**1d**) with *n*-butanol in the same five solvents (MTBE, *n*-octane, acetonitrile, THF, toluene) in presence of several lipases was performed. Most lipases (Cal-A, PPL, AK, CRL/AYS Amano and PS) were inactive. The obtained results indicated the good *R*-enantioselectivity of Cal-B, whereas MML (lipase from *Mucor miehei*) presented opposite preference (Scheme 2). The secondary hydrolysis reaction (caused by the residual water from the enzyme preparation (shell+adsorbant)) with both lipases showed good *R*-enantioselectivity. The inverse *S*-selectivity of lipases was already reported in the case of lyophilized mycelia of *Aspergillus oryzae* in the resolution of *(R,S)*-Flurbiprofen.[33,32] Moreover, MML was described as *S*-selective in the esterification of racemic naproxen with methanol.[34,33] In our case, the process catalysed by MML is a parallel/divergent resolution, allowing the preparation of optically enriched (*S*)-ester, which can be converted subsequently in the (*S*)-acid, and of (*R*)-ester with good conversions.

Based on these results, further other nucleophiles (methanol, ethanol, *n*-octanol and *n*-cetylic alcohol) were tested for the alcoholysis of all three bulky racemic esters (*rac*-**1c-e**) in presence of these two lipases with opposite enantioselectivity in the same solvents. The most relevant obtained results are presented in Figure B3.5 (conversion) and Figure B3.6 (enantiomeric excesses). In almost all cases the optical purity of the (*R*)-ibuprofen obtained by secondary hydrolytic reactions was high, while the enantiomeric excesses of the formed (*S*)- or (*R*)-ester and of the remained (*R*)- or (*S*)-ester were moderate to good.

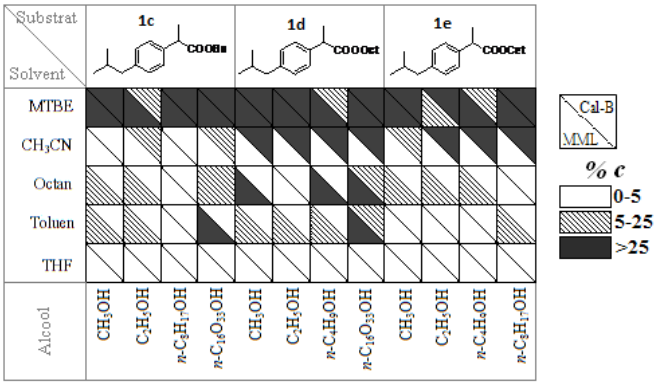


Fig. B3.5. Chemical conversion in the alcoholysis of racemic esters (*rac*-**1c-e**) with various alcohols in presence of two lipases with opposite enantioselectivity: Cal-B si MML

Fig. B3.6. Enantiomeric excess of products obtained by alcoholysis of racemic esters (*rac*-**1c-e**) with various alcohols in presence of two lipases with opposite enantioselectivity: Cal-B si MML

Concerning the obtained conversions, MTBE is the most proper solvent for the alcoholysis of all three esters with MML, excepting the ethanolysis of the cetyl ester (**1e**)

wich undergoes similarly and slowly both in MTBE and *n*-octane. When Cal-B was used as catalyst, MTBE was also convenient in most of the cases. Some exceptions were observed in the case of butanolysis of octyl- and cetyl ester (**1d-e**) with Cal-B, when acetonitrile proved to be most convenient and in the case of ethanolysis of butyl ester (**1c**), when the reactions are slowly both in MTBE and acetonitrile. *n*-Octane seems to be efficient only for the octyl ester (**1d**) and MML, while THF gave in all cases very low reaction rates.

Using the optimal conditions, next the preparative scale enzymatic resolutions were realized, obtaining both enantiomerically enriched stereoisomers of the esters. By their mild hydrolysis, both enantiomers of the target compound (*S*- and *R*-**Ibu**) were synthetized in enantiomerically enriched form.

CONCLUSIONS

1) The pharmacokinetic analysis of ketorolac plasma concentrations obtained after 10mg ketorolac dosing in healthy volunteers was done.

The Test and Reference products were administrated in a crossover designed study.

The enantioselective bioanalytical method developed and validated for usage for the biological samples from the pharmacokinetic study proved to be adequate, robust and reproducible.

In the first part of analysis, the non-compartmental pharmacokinetic analysis was used for pharmacokinetic parameters Cmax, T_{max}, ASC_{last}, ASC_{inf}, k_{el}, t½, TMR, Cl şi Vd.

There were run bioequivalence for each of the enantiomers R(+) and S(-), after test and reference product administration. Calculus present that the same speed and the size of absorption is similar, in conclusion the pharmaceutical products are bioequivalent and interchangeable.

It was confirmed the existence of a different pharmacokinetics for each enantiomer, that is justifying the separate investigation of the bioavailability and further the separate investigation of each enantiomer pharmacology , having the purpose to develop new pharmaceutical formulations using enantiopure medicinal substance.

2) It was realised the pharmacokinetic analysis of ibuprofen plasma concentrations obtained in 3 clinical studies where 400/200mg ibuprofen was administrated to healthy volunteers. The Test and Reference products were administrated in a crossover designed study. Each study was done in fasting and fed conditions, in the absence and respectively in the presence of food in the volunteer's body.

The bioanalytical method developed and validated for usage for the biological samples from the pharmacokinetic study proved to be adequate, robust and reproducible in assaying the ibuprofen in the plasma samples from fasting and fed study.

In the first part of the data analysis, it was used non-compartmental pharmacokinetic analysis. There were calculated the values of the pk parameters: Cmax, Tmax, ASClast, ASCinf, kel, t½, TMR, Cl şi Vd. The pharmacokinetic parameters values were compared referring to the pharmaceutical product dosed and respectively referring to the food effect over the pharmacokinetics. There were performed bioequivalence tests for ibuprofen after test and respectively Reference product administration. The calculus demonstrated that both speed and absorption are similar, the products are bioequivalent and interchangeable. In the same it was demonstrated a strong influence of food over the ibuprofen pharmacokinetics, especially by decreasing the speed of absorption and less by changing the size of absorption.

It was also studied the compartmental pharmacokinetics of ibuprofen. The results obtained demonstrated that the mono-compartmental model, with order 1 kinetics of absorption and order 1 kinetics of elimination, with latent time, is the best describing the

pharmacokinetics of ibuprofen. The results obtained in non-compartmental and compartmental analysis are consistent.

3) Baker's yeast mediated enantiotopic selective reduction of (benzofuran-2-yl)ketones proved to be a convenient method for preparation of optically active (benzofuran-2-yl)carbinols. Reduction of 1-(benzofuran-2-yl)ethanones yielded secondary alcohols (*S*)-**1a**–**d** with moderate to good enantiomeric excess [from 55 to 87% ee], in accordance with Prelog's rule.
4) The biotransformation with *S. cervisiae* cells of 1-(benzofuran-2-yl)-2-hydroxyethanones **3a**–**c** and 2-acetoxy-1-(benzofuran-2-yl)ethanones (**4a**–**c**) provided both enantiomeric forms of diols (*S*)-**5a**–**c** and (*R*)-**5a**–**c** in high enantiomeric purity [from 84 to 93% ee]. From the biotransformation of the acetoxymethyl ketones **3a**–c, the alcohols (−)-**5a**–**c** were exclusively isolated, indicating that the benzofuran-2-yl derivatives are good substrates of the hydrolases also present in the baker's yeast cells. According to our expectations, these alcohols had the opposite sign of specific rotation than those (+)-**5a**–**c** obtained from the biotransformation of the hydroxymethyl ketones **4a**–**c**.
5) All possible enzymatic routes involving the ibuprofen esters with long chain alcohols *rac*-**Ibu** as product or substrate were studied, with the aim to obtain the *S* enantiomer. Two lipases with opposite enantioselectivity, lipase B *C. antarctica* and lipase from *Mucor miehei* were identified. Both lipases are active in the alcoholysis of racemic *n*-octyl ester with fatty alcohols, in various organic solvents.

Selective references

[1] L. Pasteur, *Compt. Rend. Acad. Sci.* **1858**, *46*, 615–18; L. Pasteur *Compt. Rend. Acad. Sci.* **1860**, *51*,298–99.
[2] W. E. Bachmann, W.Cole, A. L. Wilds *J. Am. Chem. Soc.* **1940**, *62*, 824–839.
[3] B.G. Main *Problems and Wonders of Chiral Molecules*, Académiai Kiadó, Hungary, **1990**, pp. 329–343.
[4] G..C. Cotzias, P.S. Papavasiliou, R. Gellene *NewEngl. J. Med*. **1969**, *280*,337–345.
[5] P. S. Portoghese, *Ann. Rev. Pharmacol*. **1970**, *10*, 51–76; B.V. Rama Sastry, *Ann.Rev.Pharmacol*. **1973**, 13, 253–267; E. J.Ariens, *Med. Res. Rev.* **1986**,*6*, 451–466; I. W.Wainer(Ed.*), Drug Stereochemistry: Analytical Methods and Pharmacology*, 2nded., Marcel Dekker, NewYork, **1993**; A. J. Hutt, S.C.Tan, *Drugs* **1996**, *52*, 1–12; M. Eichelbaum, A. S. Gross *Adv. Drug. Res.***1996**, *28*, 1–64; P. Jenner, B.Testa *Drug Metab. Rev.* **1973**, *2*,117–184.
[6] A. M. Krstulovic(Ed.*), ChiralSeparations by HPLC*, Ellis Horwood Limited,UK, **1989**.
[7] L. N. Mander, *StereoselectiveSynthesis*, in E. L. Eliel, S.H. Wilen, L.N. Mander (Eds.), *Stereochemistry of Organic Compounds*,Wiley-Interscience, USA, **1994**, pp. 835–990; E. Francotte, *Preparation of Drug Enantiomers by Chromatographic Resolution on Chiral Stationary Phases*, in H.Y. Aboul-Enein, I.W. Wainer (Eds.),*The Impact of Stereochemistry on Drug Development and Use*,Wiley-Intescience, USA **1997**, pp. 633–683.
[8] M.I.Tosa, C.Paisz, F.D.Irimie *Bioprocese pentru obtinerea medicamentelor si intermediarilor,* Napoca Star, Cluj-Napoca , **2007,** pp127-153
[9] K.M.Rentsch *J.Biochem.Biophys.Methods* **2002**, *54*, 1-9
[10] J.A. Tobert,V.J. Cirillo, G. Hitzenberger, I. James, J. Pryor, T. Cook, A. Buntinx, I. B. Holmes, P.M. Lutterbeck, *Clin. Pharmacol. Ther*. **1981**, *29*, 344–350;
[11] Fried J .H, Harrison I.T:, Lewis B.,Riegl J., Roocks W., Tomolonis A., Scand J.Rheumatol[Suppl]2:**1973**,7-11;PF Juby,WR.Goodwin, TWHudmya,RA Partyka, J.Med.Chem 15 ,**1972**, pp 1297-1306;JS Kaltebronn., JMedChem 16, **1973**, pp 490-493
[12] Blanchard J, Sawchuk R J, Brodie B B. *Principles and perspectives in drug bioavailability*, Basel, New York, Karger, **1979**.
[13] Munzel K. *Pharm. Acta Helv.* **1971**, *46*, 513-38.
[14] Ritschel W A. *Physicochemical and pharmaceutical properties of drugs and dosage forms influencing the results of phase I studies*. Urban&Schwarzenberg, Munchen – Wien – Baltimore, **1977**.
[15] Kurtoglua, Y.E., Mishra, P.K., Kannanb, S., Kannana, R.M. *Int. J. Pharm.* **2010**, *384*, 189–194; Karewicza, A. Zasada, K., Szczubiałka, A., Zapotocznya, S., Lach, R., Nowakowska, M. *Int. J. Pharm.* **2010**, *385*, 163–169; Onofre, F.O., Wang, Y.-J. *Int. J. Pharm.* **2010,** *385*, 104–112; Goltsova, A, Maryashkinb, A., Swatc, M. Kosinskyb, Y. Humphery-Smithe, Y., Deminc, O., Goryanina, I.ebedeva, G. *Eur. J. Pharm. Sci.* **2009**, *36*, 122–

136; Maestrelli, F., Zerrouk, N., Cirri, M., Menninia, N., Mura, P. *Eur. J. Pharm. Sci.* **2008**, *34*, 1–11; Yeon S. K., Chang, J.H., In Ae, K., Man, B.G. *Bioorg. & Med. Chem.* **2010**, *18*, 3467–3473**.**

[16] Leucuta S E. *Biofarmacie si farmacocinetica*, Editura Dacia, **2002**;

[17] Anonymous *Fed. Reg.*, May 27, **1992**, *5(102)*, 22249; S. Laganière, Current Regulatory Guidelines of Stereoisomeric Drugs: North American, European and Japanese Point of View, , in H.Y. Aboul-Enein, I.W. Wainer (Eds.),*The Impact of Stereochemistry on Drug Development and Use*,Wiley-Intescience, USA **1997**, pp. 545–564.

[18] *FDA-Guidance for Industry-Bioanalytical Method Validation*, **2001**

[19] *CHPM-Guidance on Investigation of Bioequivalence*, **2010**

[20] A.M. Marcovici, S. Bhardwaj, A. Simedru, T.Monif, *Biomed. Chrom.*,-**submitted**

[21] A.M.Marcovici, M.I.Tosa, L.Vlase, S.E.Leucuta, F.D.Irimie, *StudiaUniv. Babes-Bolyai, Chem.***2009,** *2*, 171-179

[22] Iuliani, P., Carlucci, G., Marrone, A. *J. of Pharm. and Biomed. Anal.* **2010**, *51*, 46–55; Ilisz, I., Berkecz, R., Antal, P. *J. Chromatogr.A* **2009**, *1216*, 1845–1860; Pyka, A. *J. Liq. Chromatogr. Relat. Technol.* **2009**, *32(5)*, 723-731.

[23] M. Kusakabe; Y. Kitano; Y. Kabayashi; F. Sato *J. Org. Chem.* **1989**, 54, 2085; M. Zaidlewicz; A. Chechlowska; A. Prewysz-Kwinto.; A. Wojtczak *Heterocycles* **2001**, 55, 569-577.

[24] C. Paizs; M. Tosa; C. Majdik; P. Moldovan; L. Novak; P. Kolonits; A. Marcovici; F.D. Irimie; L. Poppe *Tetrahedron*: *Asymmetry* **2003**, *14*, 1495–1501.

[25] L.C. Bencze; C. Paizs; M.I. Tosa, F.D. Irimie, J. Retey *ChemCatChem* **2011**, *3*, 343 – 346.

[26] Evans, A.M. *J. Clin. Pharmacol.* **1996**, *36*, 7S-15S

[27] K. M. Williams; R. O. Day; R. D. Knihinicki; A. M. Duffield *Acta Pharm. Toxicol.* 1986, **59**, 192–192

[28] V. Gotor-Fernandez; E. Busto; V. Gotor *Adv. Synth. Catal.* **2006**, *348*, 797–812; A.K. Prasad; M. Husain; B.K. Singh; R.K. Gupta; V.K. Manchanda; C.E. Olsen; V.S. Parmar; *Tetrahedron Lett.* **2005**, *46*, 4511–4514.

[29] Lane, C., Boeren S., Vos, K., Veeger, C. *Biotechnol. Bioemg.* **1987**, *30*, 81-87

[30] K. Engström; J. Nyhlen; A. G. Sandström; J. E. Backväll *J. Am. Chem. Soc.* **2010**, *132*, 7038–7042; A. G. Sandström, K. Engström; J. Jyhlen; A. Kasrayan; J.E. Bäckvall *Protein Eng., Des. Sel.* **2009**, 22, 413–420

[31] NOI CU FATTY ACIDS ART DIN 2012

[32] P. Spizzo; A. Basso; C. Ebert; L. Gardossi; V. Ferrario; D. Romano; F. Molinari *Tetrahedron* **2007,** 63, 11005-11010

[33] R. Morrone, N. D'Antona, D. Lambusta, G. Nicolosi *J. Mol. Catal. B: Enzymatic* **2010**, *65*, 49-51