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TRANSPORT OF NSAIDs ACROSS THE BLOOD-BRAIN BARRIER *IN VITRO*

Diploma thesis

in cooperation with

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Abstract

The migration of substances between the blood circulation and the central nervous system (CNS) is regulated by the blood-brain barrier (BBB). Small, lipophilic molecules such as carbon dioxide, oxygen or ethanol can pass the BBB by passive, transcellular diffusion, while the paracellular transport of hydrophilic substances is restricted by intercellular tight junctions. Due to accessory transport systems, the BBB is able to regulate specifically the permeation of substances (e.g. nutrients) (Ballabh et al., 2004).

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most commonly used substances world-wide, yet little is known about their ability to cross the BBB. Since NSAIDs may exhibit CNS side effects including dizziness, headaches and drowsiness we sought to study the transport of several NSAIDs (celecoxib, diclofenac, ibuprofen, lornoxicam, meloxicam, piroxicam and tenoxicam).

Both single studies and group studies were carried out applying either a single substance or several substances simultaneously across the BBB *in vitro* model based on the human cell line ECV304. The permeability data were normalized to the internal standards diazepam and carboxyfluorescein to account for cell layer's variabilities.

According to our studies, it was confirmed that crossing of ibuprofen and diclofenac through the BBB is strongly dependent on their free fraction in plasma. Comparisons with other studies carried out with cell line Caco-2 pointed to a relation between an unknown efflux system and the decreased influx of ibuprofen into the brain. Carboxyfluorescein used as a paracellular marker showed some interactions with other substances (ibuprofen, diclofenac) and thereby may not be the perfect internal standard. Addition of transporter blocker probenecid did not show any effects on ibuprofen transport, but significantly inhibited the transport of tenoxicam. Finally, the second used blocker verapamil revealed a possible relation to celecoxib. Overall, our findings suggested that the transport of ibuprofen, diclofenac, carboxyfluorescein, tenoxicam and celecoxib is supported by still unknown transport systems.

Furthermore, these results link the individual permeability coefficients with the incidence and severity of CNS side effects of the individual substances and may guide future NSAIDs drug design.

Abstrakt

Přechod substancí mezi krevním řečištěm a centrálním nervovým systémem (CNS) je regulován hematoencefalickou bariérou (HEB). Malé, lipofilní molekuly jako jsou oxid uhličitý, kyslík nebo etanol mohou procházet přes HEB pomocí pasivní, transcelulární difuze. Naproti tomu schopnost paracelulárního přechodu hydrofilních látek je omezena intercelulárními tight junctions. Díky doprovodným transportním systémům je HEB schopna specificky regulovat transport substancí (např. živin) (Ballabh et al., 2004).

Nesteroidní antiflogistika (NSAIDs) jsou v dnešní době hojně užívaná po celém světě, ale prozatím je jen málo známo o jejich schopnosti procházet přes HEB. Vyvoláváním nežádoucích účinků v CNS, mezi ně počítaje např. bolesti hlavy, závratě nebo ospalost, NSAIDs vzbudily zájem o bližší prozkoumání jejich transportních mechanizmů (pro naše studie vybrané látky: celekoxib, diklofenak, ibuprofen, lornoxikam, meloxikam, piroxikam a tenoxikam).

Studie byly provedeny jak jednotlivé tak skupinové, s použitím jedné nebo více substancí současně. K pokusům byl používán *in vitro* model hematoencefalické bariéry vytvořený pomocí humánní buněčné linie ECV304. Protože buněčná vrstva byla pokaždé značně variabilní, byly přidávány vnitřní standardy – diazepam a carboxyfluorescein – a ve vztahu k nim byla data průchodnosti jednotlivých substancí normalizována.

Na základě našich studií bylo potvrzeno, že přechod ibuprofenu a diklofenaku před HEB je silně závislý na jejich volné frakci v plazmě. Srovnání s jinými studiemi provedenými na buněčné linii Caco-2 poukázaly na vztah mezi neznámým efluxním systémem a omezeným vstupem ibuprofenu do mozku. Carboxyfluorescein sloužící jako paracelulární marker vykazoval interakce s několika substancemi (ibuprofen, diklofenak) a proto není příliš ideálním vnitřním standardem. Přídavek blokátoru transportních systémů probenecidu nevykázal žádný vliv na přenos ibuprofenu, ale výrazně omezil transport tenoxikamu. Také druhý použitý inhibitor verapamil odhalil možný vztah k celekoxibu. Celkově naše poznatky naznačují, že transport ibuprofenu, diklofenaku, carboxyfluoresceinu, tenoxikamu a celekoxibu je podporován zatím stále ještě neznámým transportním mechanizmem.

Mimo jiné získané výsledky vypovídají o vztahu mezi rozdílnými koeficienty permeability a výskytem nežádoucích účinků na CNS jednotlivých látek. Tím lze do budoucna pomoci při usměrňování dalšího vývoje NSAIDs.

Prohlášení

Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracovala samostatně. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány.

Contents

1 INTRODUCTION

1.1 Discovery of the blood-brain barrier

The discovery of the blood-brain barrier (BBB) is attributed to the German scientist Paul Ehrlich (1854-1915), who was active in fields as immunology, haematology and chemotherapy. In 1908, he received the Nobel Prize in Physiology and Medicine. He made experiments, in which he used to dye structures with aniline derivatives. He observed after injecting dyes intravenously into an animal body that he could see all organs except the brain. At that time, he still did not know about the BBB and he just thought that the brain was less able to accept the colour (Ribatti et al., 2006).

Ehrlich´s student Edwin Goldmann (1878-1956) made further experiments, in which he injected the dye directly into the spinal fluid. Surprisingly he found out that only the brain was dyed and that the colour did not enter into the peripheral body parts. These experiments pointed to an idea that there may be some kind of barrier between the central nervous system and the rest of the body.

In 1900 Lewandowski used for the first time the name „blood-brain barrier“ to describe the mysterious barrier between blood and the brain.

But the very first concept of the blood-brain barrier (called also haematoencephalic barrier) was proposed by Latvian biochemist and physiologist Lina Stern (1878-1968) in 1921. She carried out the pioneering experiments with the BBB.

Lately in the 1960s with developing of the scanning electron microscope, the BBB could be visualised.

Although since that the science was moving fast, during the next decade all experiments were made only *in vivo* or *in situ* (Abbott, 2005a). Finally, in 1970s first brain capillaries were isolated and so available for *in vitro* studies to extend the knowledge about the BBB (Joó and Karnishina, 1973).

1.2 The Blood-Brain Barrier (BBB)

The central nervous system (CNS) is the most important organ of the human body. It has the major control function, which is essential for the normal body activities and surviving. The brain is composed from different parts including some protective mechanisms, which are necessary to keep the homeostasis of the brain. Among these, there is the arachnoid membrane of the meninges, the blood-brain barrier (BBB) and the choroid plexus epithelium that secretes the cerebrospinal fluid (Abbott, 2005b). The BBB has the shortest diffusion distance from the capillaries to neurons (10-15 μm), which makes the BBB the most important interface between the blood and the brain.

The BBB forms a physical restriction, regulates the transport of different substances and also shows metabolic activities. The BBB alone creates the largest surface area (including just the brain parts) with 12-20 m^2 by usual brain weigh of 1.3 kg. The BBB has the major influence of the access of substances from the blood to the CNS and vice versa. Without this barrier the migration of chemical substances, toxins and bacteria would not be controlled and that would let many dangerous substances enter the CNS, endanger the physiological functions of the CNS and maybe also cause damages. Although bacterial infections in the brain are very rare, if they appear they cause serious diseases as for example bacterial meningitis, which are difficult to treat.

In addition to the restriction of bacterial migration across the BBB, one further main function of the BBB is to regulate the crossing of essential nutrients as well as the movement of ions and fluids and the efflux of many waste products. Because of these restrictive mechanisms the BBB protects the brain from ionic fluctuations caused by digestion, respiration or exercise, which can have a negative influence on the neuronal signalling.

There should not be forgotten that the BBB also separates transmitter pools in the brain from those in the periphery so they cannot influence each other.

Nevertheless, there are some parts of the brain, which are not protected by the BBB. Two regions - area postrema and posterior pituitary area – are missing the protection and the capillaries here are fenestrated as those in the rest of the body.

The main components of the barrier are the capillary endothelial cells, which lack fenestrations, the pinocytic vesicular transport is sporadic and the tight junctions are more extensive. These properties differ the BBB endothelial cells from endothelial cells in peripheral body parts. The capillary endothelial cells encircle the capillaries and are connected to each other with tight junctions to increase the tightness of the paracellular space. Additionally, the basal lamina, pericytes and perivascular astrocyte end-feet are important for a complete functional BBB (Figure 1.1). Further, there are more other cell types present in the perivascular space as e.g. smooth muscles (in larger vessels) or perivascular macrophages. All these components make a fair assumption to be essential for the BBB to keep its integrity, stability and the right physiological function (Abbott, 2006).

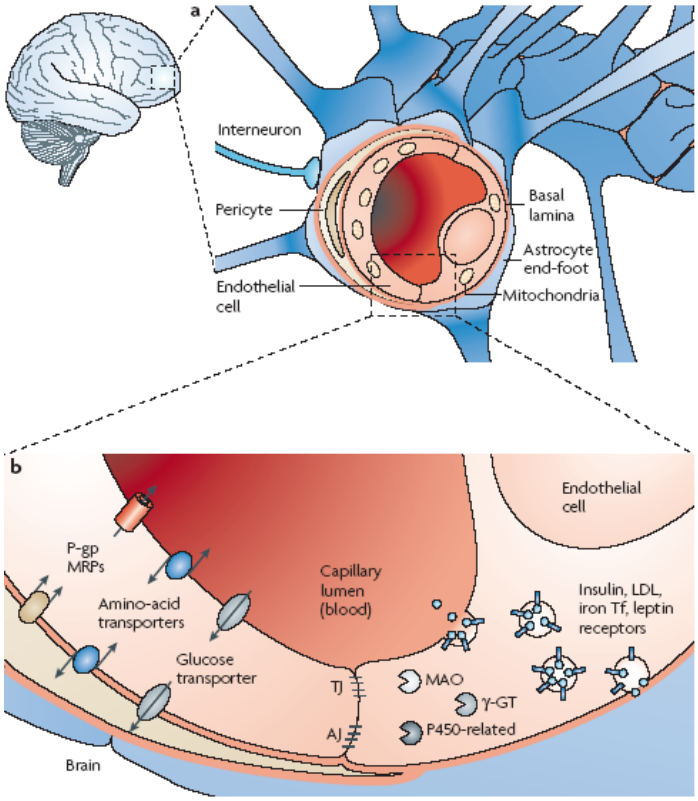


Figure 1.1 Scheme of a blood capillary
Picture „a“ shows a blood capillary encircled by endothelial cells, basal lamina and pericytes and finally enclosed by astrocyte end-feet and interneurons.
The lower picture „b“ describes enlarged part of the capillary and endothelial cells. At the contact of two endothelial cells tight junctions (TJ) and adherens junctions (AJ) are symbolically represented. There are also shown some transmembrane transport systems for amino acids, glucose and for P-gp and further, some receptor-mediated endocytosis systems for leptin, insulin, iron transferrin etc. Source (Cecchelli et al., 2007)

1.2.1 Transport systems

Because of the very tightly sealed (caused by tight and adherens junctions) paracellular space, many substances are forced to enter or leave the CNS through the transcellular route, where many uptake and efflux transporters are available for this purpose. Especially hydrophilic substances are impeded to cross the BBB and enter into the CNS.

Small lipophilic molecules as e.g. oxygen, carbon dioxide or ethanol, can diffuse across the plasma membrane freely along their concentration gradient (Ballabh et al., 2004).

Other substances as nutrients (amino acids, glucose, organic anions and cations etc.) are transported into the CNS via special transport mechanisms. Up to now more than 20 uptake transport systems were identified. Some of these uptake transporters enquire energy in form of ATP (e.g. Na^+, K^+-ATPase), some of them work as a facilitating diffusion transfer system (e.g. GLUT-1 for glucose) and are energy-independent. Other substances can pass the BBB only by coupling with other molecules, which is called secondary active transport (e.g. Na^+-coupled) (O´Kane et al., 1999).

The localisations of transporters also differ from type to type. Some of them are available on both sides of the membrane (luminal and apical), some are located just on one side. The real purpose of the apical/luminal distribution of transporters is up to now still unclear.

According to the observations, it is known that the transport systems do not only enable the passing of substance from the blood into the brain, but some of them can also have an efflux function.

Finally, it is necessary to mention families of transporters, which ensure only the transport out of the CNS, e.g. the ABC-transporter family including P-glycoprotein (ABCB1), the Multidrug Resistance Related Proteins (MRPs, ABCC1, 2, 5) and the Breast Resistance Proteins (BCRP, ABCG2). Until now, the role of these proteins has not been explained satisfactorily, but it is sure that they can restrict the entrance of toxins and other agents into the brain.

The above introduced transport-ways enable passing of substances just up to a defined molecular weight (< 1000 MW). Thus, there must be some other transport possibilities for larger molecules as proteins. Their uptake can be mediated by

transcytosis, of which two different types of mechanisms were identified. The first type is the receptor mediated transcytosis (RMT), which supposes a specific interaction with cell membrane receptor (e.g. transport of insulin, leptin or iron transferrin) (Pardridge, 2005). The other type – adsorptive mediated transcytosis (AMT) – is less specific and requires only binding of cationic molecules to negatively charged endothelial glycocalyx. Anyway, this type of transport system is less active than in the periphery (Abbott, 2005b). The scheme describing transport systems possibilities is shown in Figure 1.2.

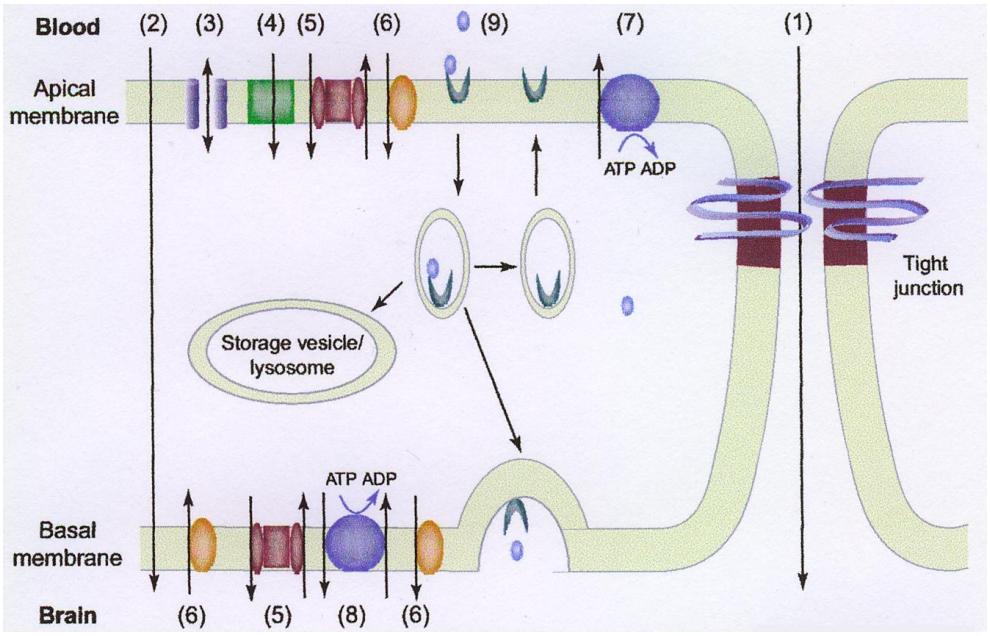


Figure 1.2 Transport systems

Different transport systems: (1) Paracellular pathway for small polar molecules; (2) Passive diffusion; (3) Cation channels; (4) Ion symports; (5) Ion antiports; (6) Facilitated diffusion (GLUT-1); (7) Active transport (P-gp); (8) Active antiport transport; (9) Endocytosis. Source (Huber et al., 2001)

1.2.2 Tight junctions

Tight junctions (TJs) are the main components sealing the paracellular space, which restrict free migration of substances to or from the CNS. TJs are composed of different proteins, which form a complex in the membrane. Namely, belong to these proteins claudin, occludin, junctional adhesion molecules and also many other accessory proteins as e.g. zonula occludens (ZO-1, ZO-2, ZO-3), cingulin etc. The proof of the very high and effective tightness by the TJs is shown by the transendothelial electrical resistance (TEER), which reaches in the brain values over 1000 Ohm.cm^2 in comparison to the peripheral parts, where the TEER values are only between 2 and 20 Ohm.cm^2 (Abbott et al., 2006).

Claudins form a huge protein family with at least 24 members, which have been identified until now. Claudins are phosphoproteins, build the major part of the TJs and possess four transmembrane domains. The COOH-terminal domain is bound to ZO-1, ZO-2 and ZO-3 in the cytoplasm. It was found out, that claudin-1 and -5 together with occludin are present in the TJs of the BBB and so participate on building its tightness.

Occludin is also a phosphoprotein and is larger than the claudins. It has four transmembrane domains between its COOH-terminal on one end and NH_2-terminal on the other end in the cytoplasm. The paracellular space is restricted by the two occludin's extracellular loops together with claudin's loops. Also here, the terminal domains are associated to the ZO proteins in the cytoplasm.

Both claudins and occludin build the most important components of the TJs and are essential for forming of the BBB. According to this statement, it was proved that disturbance of physiological functions of the BBB was connected with the loss of occludin 1 (Liebner et al., 2000b).

The junctional adhesion molecules (JAMs) are the next important type of membrane proteins of the TJs. These JAMs belong to an immunoglobulin superfamily and by now three subtypes were discovered. The single transmembrane domain formed by JAMs, has two immunoglobulin-like loops, which are made by two disulfide bonds. JAMs are involved in monocyte transmigration processes through the BBB and are necessary for cell-to-cell adhesion (Ballabh et al., 2004).

Proteins from membrane-associated guanylate kinase-like proteins (MAGUKs) are part of the cytoplasmatic accessory proteins. Zonula occludens proteins (ZO-1, ZO-2,

ZO-3) and cingulin belong to this group. One of the three PDZ domains (PZD1) of the ZOs allows them to connect directly to the COOH-terminal of claudins. Occludin as well as JAMs makes also some connections with ZO-proteins but with other domains than claudins. Then actin, the primary cytoskeleton protein, connects with ZO-proteins as well and plays a very important role in this complex.

Cadherins, belonging to adherens junctions, are another type of protein, which is concerned in the restriction of the paracellular space. They form adhesive contacts between cells. Cadherins are in touch with actin via catenins, which are intermediary proteins.

Restriction of the paracellular space with all proteins taking part in it is illustrated in Figure 1.3.

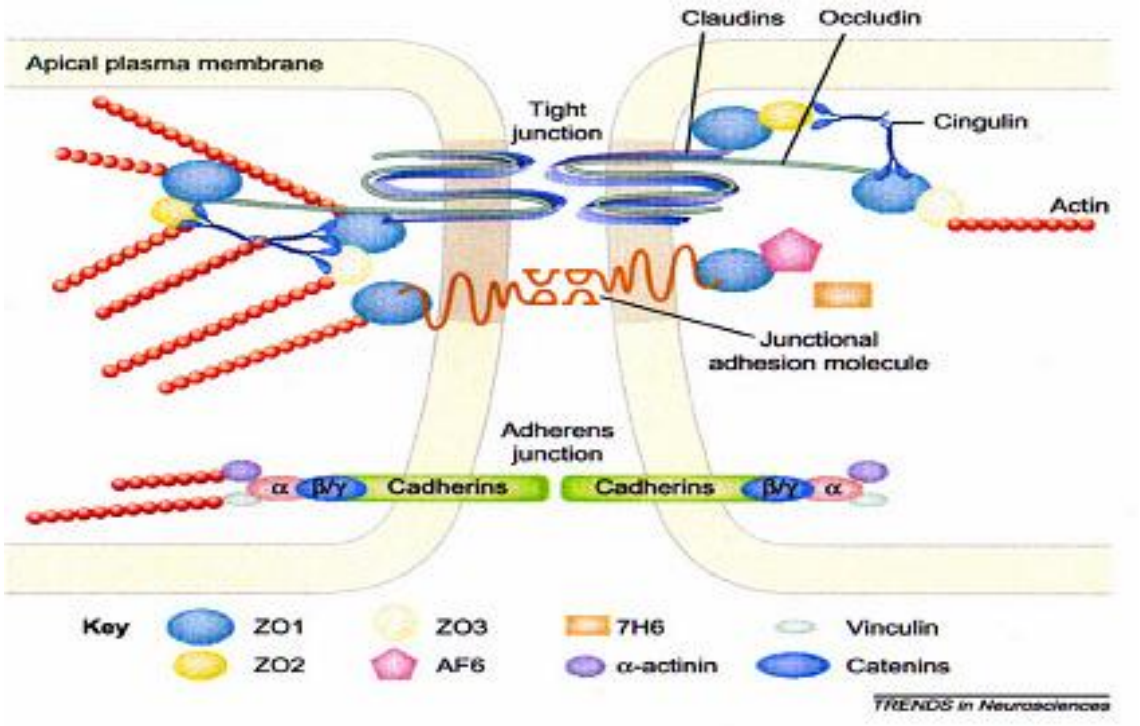


Figure 1.3 Scheme of the paracellular space
The picture shows the paracellular space between two endothelial cells of the brain capillary, which is restricted by tight junctions (TJs), junctional adhesion molecules (JAMs) and adherens junctions (AJ). The picture describes in detail the components of the TJs – claudins and occludins with their transcellular loops and further connections to zonula occludens (ZO-1, ZO-2, ZO-3), cingulin and actin. It is shown that JAM proteins, building two immunoglobulin-like loops in the paracellular space, are also connected to ZO-proteins. The last part of the restriction of the paracellular space is ensured by AJ, compound of cadherins joint via catenins to actin fibres. Source (Huber et al., 2001)

1.2.3 Astrocytes

Astrocytes (belonging to glia cells) have a close connection to the endothelial cells by their astrocytic end-feet. Their close position to the endothelial cells is important. Astrocytes secrete several chemical substances, especially growth factors, which can modulate the expression of transporters (P-gp, GLUT1 etc.) and up-regulate the tightness of the BBB. For improving growth conditions, cells were co-cultured with astrocytes or at least astrocytic factors were added to growth media (Neuhaus et al., 2008b).

1.2.4 Other cell types influencing the BBB properties

The BBB must be seen as a complex of many proteins and different cell-types, which cooperate together and all of them play an irreplaceable role in the BBB unit. That is why the whole complex is sometimes called as „neurovascular unit". Several other components can influence the properties of the BBB in addition to the endothelial cells and astrocytes. For example, the basal lamina, pericytes, perivascular macrophages, neurons and smooth muscle cells can modulate the BBB.

The basal lamina is an extracellular matrix layer and surrounds endothelial cells. Its role is especially anchoring the cell-cell interaction and transmitting their signals. Due to its tightness, it provides also barrier function and mechanical support.

In some places pericytes are incorporated into the basal lamina. These pericytes fix the structural stability of the microvessels. This view is supported by many studies, which have shown that endothelial cells cultivated without pericytes were inclinated to apoptosis more often (Ramsauer et al., 2002). Their role in angiogenesis was also investigated (Balabanov and Dore-Duffy, 1998; Hirschi and D`Amore, 1997). Pericytes express on their surface many receptors for chemical mediators, which can influence the permeability and flexibility of the blood vessels (e.g. vasopressin, angiotensin II, endothelin-1 etc.) (Ballabh et al., 2004). Unfortunately, the pericytes are still the least studied component of the BBB.

Neurons end on or close to the blood vessels and from this position they can influence the vessels by releasing transmitters (Abbott, 2005b).

Smooth muscles are occupying pericytes within an expanded collagenous extracellular matrix. They can be found especially in larger vessels, thus, they play a minor role at the BBB in the capillaries.

1.2.5 Relation between the BBB and diseases

As described above, the BBB is a functional unit, where every single damage of any substance can cause changes. For example, if the tight junctions of the BBB open, the permeation of substances would increase and pathogens can permeate in direction to the brain. This can occur in many different diseases as e.g. during inflammation, brain oedema, hypoxia-ischemia, stroke, trauma, pain etc. (Abbott et al., 2006).

It was shown that some types of tumour – adenocarcinoma, glioblastoma – contribute to the down-regulation of expression of some proteins and so indirectly to contribute to the breakdown of the BBB (Liebner et al., 2000a).

It was also reported that expansion of the inflammatory mechanism plays a role during Alzheimer‘s disease, Parkinson‘s disease, epilepsy, multiple sclerosis and HIV-associated dementia. Due to inflammation and production of several toxins, the breakdown of the BBB can occur in these diseases (Ballabh et al., 2004).

Different diseases affect different parts of the BBB, but at the end the result is always the same: pathological conditions. In spite of, many studies were made, still not all connections between diseases and breakdown-mechanisms of the BBB are known yet. Further investigations and observations should open new perspectives for the understanding and lead to improvements of the treatment.

1.2.6 Potential brain targeting

The BBB represents a huge challenge for scientists designing drugs, which can be delivered into the brain and though to overcome the BBB.

During the pathological affection BBB functions are down-regulated or already down-broken and this allows many drugs to pass into the CNS easily. But what if the restriction by the BBB is not influenced? This creates an interest to find the right delivery vehicle for drugs.

Up to now e.g. use amino acid carriers for transporting different kinds of drugs as e.g. L-dopa or gabapentin, are used very successfully. These carriers fill the request of solubility and accepting of large hydrophobic molecules at once.

There are three main requests on the delivery strategy to bypass efflux systems: 1) to reduce the affinity for efflux transporters 2) blocking P-gp function or 3) cover drugs so they cannot be detected by P-gp efflux system (Abbott, 2005b). Although not all of these methods are perfect, they influence the process of brain targeting and are contributing to make further steps in the development. Other methods to overcome the BBB are the opening of the BBB by induction of hyperosmolarity with mannitol, usage of Trojan horses (antibody technology) or receptor (targeting transferrin, insulin, ApoE receptors) and adsorption mediated transcytosis.

1.3 Non-steroidal anti-inflammatory drugs (NSAIDs)

The increase of non-steroidal anti-inflammatory drugs (NSAIDs) usage is enormous in these days world-wide, especially because of their general anti-inflammatory, antipyretic and analgetic effects. The fact that most of them belong to the „over-the-counter“ (OTC) drugs, which someone can get without prescription, supports the use even more. So there is no wonder that the request to study their passage across the blood-brain barrier (BBB) is increased, especially with regard to possible effects of long-term therapy use.

1.3.1 Common characteristics

NSAIDs are generally acids (diclofenac, ibuprofen) or at least they have an acidic vinylogous group (meloxicam, lornoxicam, piroxicam, tenoxicam). Their molecular weight is between 206 and 390 g/mol. Table 1.1 summarises the chemical structures, molecular weights and plasma half life times of the NSAIDs used in the experiments.

NSAIDs inhibit the enzyme cyclooxygenase (COX), which was already discovered in 1970s by Vane (Vane, 1971). COX is an important enzyme in the whole process of the formation of prostanoids (prostacyclins, prostaglandins, thromboxanes). These prostanoids influence many physiological processes in the human body and thereto under the pathologic circumstances they cause progress of fever, pain and inflammation.

The pathway starts with arachidonic acid, which is released from the phospholipids of the cell membrane by means of the enzyme phospholipase A_2. The conversion of arachidonic acid is catalysed by COX enzymes. The first product of the conversion is prostaglandin G_2, which is further converted to other prostaglandins or to prostacyclins or to thromboxane A_2. There is also another possibility of the conversion of arachidonic acid. It can also be transformed into leukotrienes. They play an important role in asthma bronchiale and allergic reactions. For more details see the scheme of the metabolic pathway (Figure 1.4).

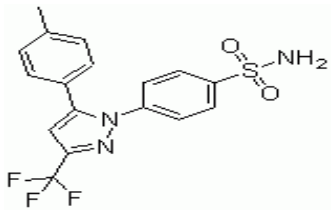
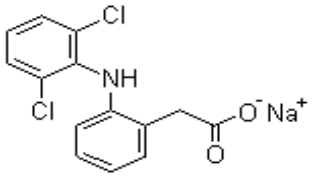
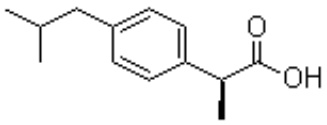
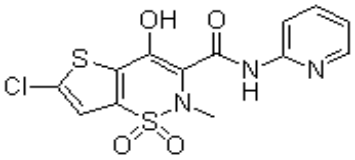
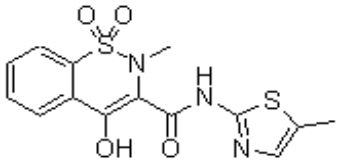
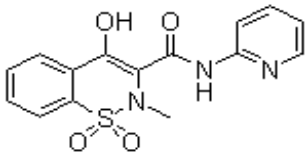
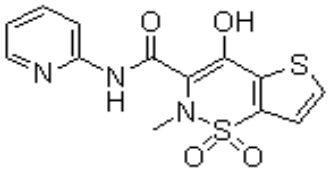
Substance	Structure	MW [g/mol]	Half life [hours]
Celecoxib		381.37	11
Diclofenac (sodium salt)		296.15 318.13	1 – 2
Ibuprofen		206.28	1 – 2
Lornoxicam		371.82	3 – 6
Meloxicam (sodium salt)		351.40 378.38	15 - 20
Piroxicam		331.35	50 - 80
Tenoxicam		337.38	60 - 70

Table1.1 Overview of NSAIDs used in the studies.
Source (Lincová, Farghali et al., 2007; Olkkola et al., 1994; Guentert et al., 1987)

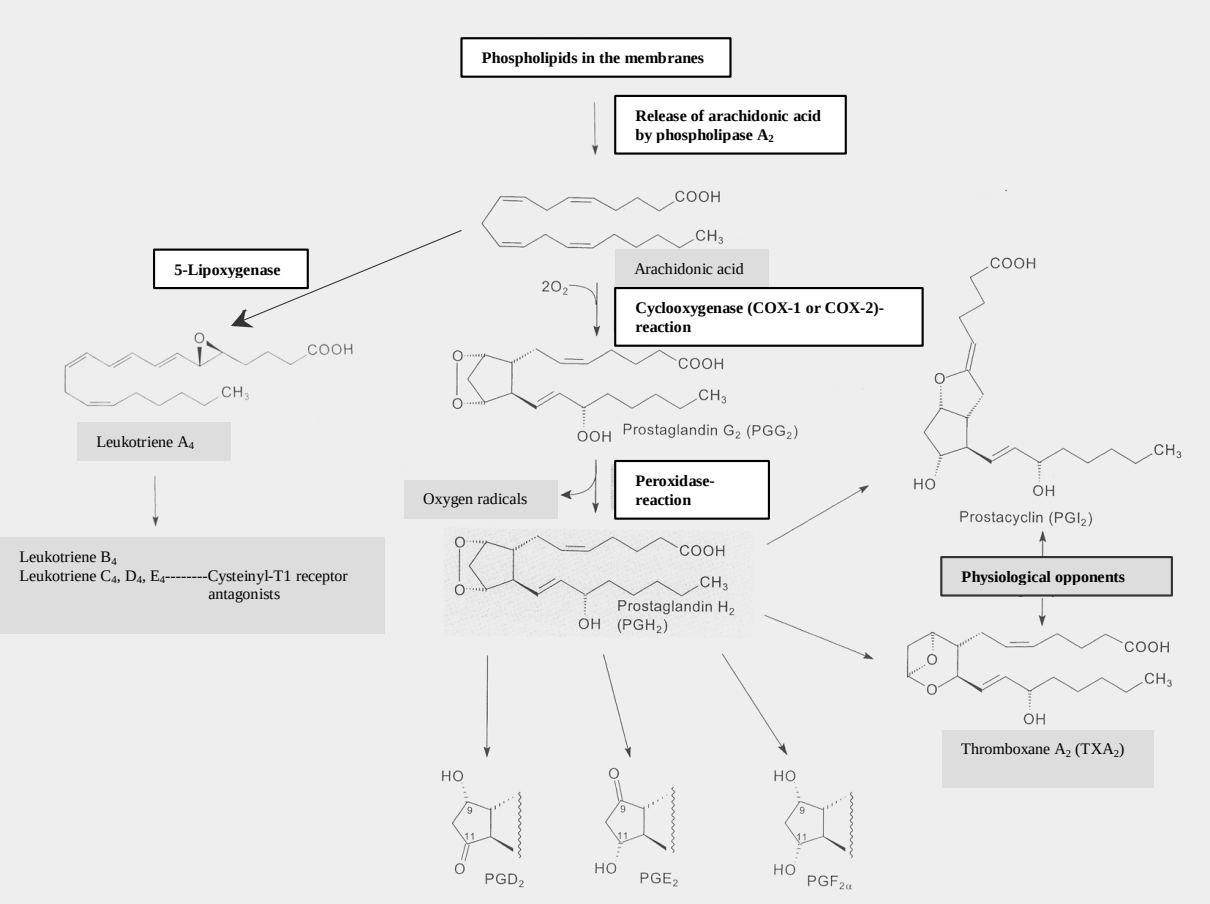


Figure 1.4 Conversion of arachidonic acid
The picture shows the pathway of conversion of arachidonic acid into leukotrienes, prostaglandins and thromboxanes with enzymes, which enable converting of the substances. Source (Steinhilber et al., 2005)

Prostacyclins and thromboxanes play a major role in the aggregation process of platelets. Prostacyclins are released from the endothelial cells and inhibit the aggregation. In contrast to this, thromboxanes are released from platelets and support their aggregation. Under normal circumstances this process is in balance of both substances.

In the human body it is differentiated between two major isoforms of the enzyme cyclooxygenase (Hla and Neilson, 1992). COX-1 is the more constitutive variant, which could be found in all human cells. It is important for the regulation of physiological and homeostatic functions. On the other hand, COX-2 is an induced isoform, which can be synthesized after influence of inflammatory substances (TNF-alpha, IL-1, IL-2 etc.). COX -2 produces prostanoids, which propagate further the local inflammation, pain and fever. The differentiation between both COX functions is not that easy. It was found out that also COX-2 could have some physiological functions, especially in the CNS, kidneys and digestive tract. On the other hand, also COX-1 plays some roles in the process of

inflammatory, fever and pain (Garavito et al., 2002). In the last years, a third isoform of the enzyme - COX-3 - was identified. This variant is located especially in the heart, CNS and maybe, also in other tissues, but the exact effects after inhibiting this enzyme have not been examined yet.

In relation to these two major isoforms of COX (COX-1 and -2), the NSAIDs show also different COX-isoform dependent specificities. The first group of investigated NSAIDs is a group with no absolute specificity to one of the two COX enzymes. Ibuprofen, piroxicam, lornoxicam and tenoxicam belong to this group and prefer more COX-1 to COX-2. Meloxicam and diclofenac have COX-2 preferences, but also inhibit COX-1. On the other hand, there are substances just with COX-2 specifity, where coxibs belong to (celecoxib).

As it was mentioned before, NSAIDs are used for the reduction of fever, pain and to stop inflammatory process. The controlling of pain is provided by a peripheral mechanism and partly by influencing the feeling of pain in the CNS, but they cannot affect the visceral pain (Burian et al., 2005). Their positive attribute is that they can be used by chronic pain without developing addiction on them. Decreasing of fever is very fast and effective, but they do not influence the normal body temperature. In addition, NSAIDs act also anti-inflammatory. They can stop the acute phase of inflammation, but not the chronic stadium. For this feature NSAIDs are very often used in the treatment of rheumatic arthritis. However, the effects of various NSAIDs differ, not every substance can be used for the treatment in the first line. Finally, these substances are used because of their antiaggregation effects. For this, acetylsalicylic acid is very often applied in very low dosages (75 – 150 mg). This effect is reached due to the inhibition of COX-1. According to this fact, some NSAIDs are missing this specific activity (e.g. coxibs).

1.3.2 Side effects

By using NSAIDs we should always have their adverse effects on mind. With regard to their low specificity to COX enzymes, it can be said that almost all substances have the same side effects – more or less. Because of the inhibition of COX-1, which is involved in protection process of the stomach mucosa, side effects as e.g. ulcerates, erosion in digestive tract, nausea, gastritis, bleeding, diarrhoea or constipation can occur. The blocking of thromboxanes synthesis in thrombocytes leads to decrease of aggregation process and so to the increase of bleeding. NSAIDs can influence also the renal function, which is normally controlled by prostaglandins and prostacyclins. Using NSAIDs can lead from down-regulation of the renal function until their total insufficiency. This pathological process can be completed with hyperkalaemia, retention of natrium, water and can cause additionally oedemas. The inhibition of the conversion of arachidonic acid by COX may increase elevated production of leukotrienes, which can induce bronchoconstriction or asthma bronchiale, especially by predisposed persons. It was believed that coxibs with their selective inhibition of COX-2 would not have so many side effects, which are caused especially by COX-1 inhibition. But the reality was quite disappointed. There is a huge danger of thromboembolism and cardiovascular (heart attack) and cerebrovascular (stroke) complications. Nevertheless, there is one positive property of COX-2 blockers. They do not influence synthesis of thromboxanes by COX-1 and so do not cause increased bleeding danger. This knowledge is used by patients undergoing surgery while they cannot stop using NSAIDs.

Alongside these peripheral side effects, also some effects on the CNS were described as e.g. dizziness, headaches and drowsiness, depressions, hearing and visual impairment, tinnitus etc. (Lincová, Farghali et al., 2007). NSAIDs have to cross the BBB in order to cause these adverse side effects in the CNS. Thus, the investigation of the NSAID permeability of the BBB is of high importance. These side effects may also restrict their use by different polymorbid patients. On the contrary, the intake of ibuprofen was reported to decrease the number of Alzheimer's cases. Also in that case, the NSAIDs maybe have to permeate the BBB (Dokmeci, 2004; Parepally et al., 2006).

1.3.3 Pharmacokinetics and use of NSAIDs

All of these NSAIDs can be used orally, parenterally, rectal or also, some of them can be used locally. By per os dosage, which is most common, the substances are very good absorbed from the digestive tract. The protein plasma binding is very high, generally about 98-99 % and can cause interactions with other drugs (e.g. warfarine, oral antidiabetics, sulphonamides etc.). The distribution into other tissues and body fluids is very good. Also the transport into the CNS for several substances is remarkable. The passage through placenta can cause negative effects on the foetus. The degradation follows in liver and the active substances are metabolised into inactive substances by using isoenzymes CYP3A4 or CYP2D6. Just a very small percentage of the active substance (5-10 %) can be eliminated without any changes by stool or urine. The half lives for the oxicams are about 20-70 hours, so they can be dosed once or twice a day (Olkkola et al., 1994; Guentert et al., 1987). The half lives for ibuprofen and diclofenac are about 1-2 hours, so their use is mainly three or four times a day. More details about half lives are shown in the Table 1.1 before.

Probably the most used NSAID is ibuprofen, which is known for several years, belongs to OTC drugs and shows fewer side effects. It is used for reduction of fever, acute pain, dysmenorrhoea, pain of muscles, post-surgical and chronic pain and inflammation. Sometimes is ibuprofen combined with other drugs, e.g. with pseudoephedrine to overcome flu-like symptoms.

Further, NSAIDs are used also for treatment of osteoarthritis and rheumatoid arthritis, long-term inflammatory diseases generally and gout.

2 AIM

It was observed by patients using ibuprofen by long-term treatment of rheumatoid arthritis that they suffer less from Alzheimer‘s disease in comparison to persons with the same age without any need to use ibuprofen (or any other NSAIDs) for longer time (Dokmeci, 2004). That pointed to the fact that ibuprofen is passing the BBB and is reducing the beta-amyloid plaques in the brain (which are connected with the genesis of Alzheimer‘s disease). This finding leads to an idea to investigate the conditions under which and how is ibuprofen passing the BBB in more detail.

Considering some studies about ibuprofen the question arose if other NSAIDs could have the same potential. This was supported by the increasing use of NSAIDs during long-term treatment of diseases. Up to now, no systematical compact study including more than three substances to prove permeation abilities of NSAIDs across the BBB and to compare them in single and group studies was made.

Thereby, the aim of this diploma thesis was to investigate and compare the abilities of NSAIDs to overcome the BBB. These studies involved seven different NSAIDs (celecoxib, diclofenac, ibuprofen, lornoxicam, meloxicam, piroxicam and tenoxicam), which were tested in single studies each as well as in group studies (except lornoxicam) under different conditions. For example, the amount of applied organic solvent, the dependence on serum protein content and on the presence of astrocytic factors, varied substance group compositions and the influence of transporter inhibitors should be explored.

3 MATERIAL AND METHODS

3.1 Material

3.1.1 Cell line ECV304

The ECV304 cell line was reported as a spontaneously transformed human umbilical vein cell line, which was obtained from the European Collection of Cell Cultures. If the cells are co-cultured with rat C6 glioma cells or astrocyte conditioned medium (ACM) is used as a growth medium, they exhibit increased TEER values (Hurst, Fritz, 1996). It was found out that while co-culturing with astrocytes, the glucose transporter GLUT-1 and gamma glutamyl transpeptidase were up-regulated (Kuchler-Bopp et al., 1999). While co-culturing with C6 cells, P-glycoprotein (P-gp) and tranferrin receptor were also expressed. Typical morphology of the ECV304 is shown in the Figure 3.1. Information about used growth media is mentioned in further parts (3.1.2).

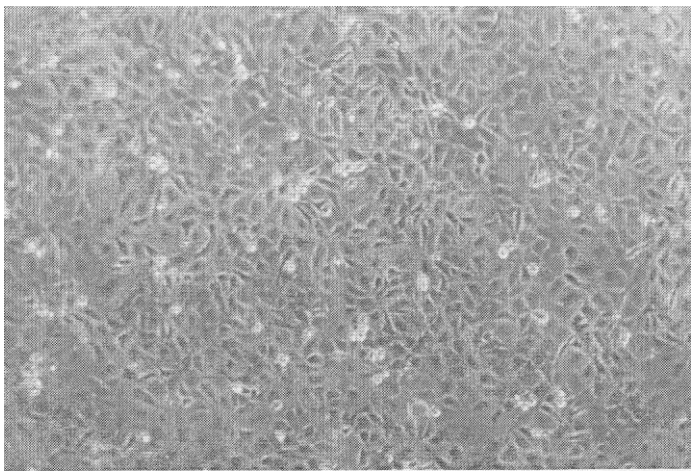


Figure 3.1 Confluent ECV304 under the light microscope.
For this cell line their cobble stone morphology is typical.

3.1.2 Media

For all experiments three types of media were used. Another special medium was used for freezing of cells.

3.1.2.1 PBMEC minus (= without) Fibronectin medium

This medium was used for general treatment of the cells as cultivating, media changing, spitting and seeding them. In the Table 3.1 exact volumes of different components of the medium are listed. The medium was always prepared under sterile conditions and was stored at 4 °C. Before use, it was pre-warmed to reach the 37 °C.

PBMEC minus Fib medium	**For 500 ml**
Ham´s F12	103.75 ml
IMDM	103.75 ml
200 mM L-glutamine	17.50 ml
10 mg/ml Holo-transferrin	250.00 µl
1000 U/ml Heparin	250.00 µl
NCS	19.00 ml
10 000U/ml Penicillin; 10 mg/ml Streptomycin	5.00 ml
250 µg/ml Amphotericin B	500.00 µl
ACM (collected supernatant of C6 medium, sterile)	250.00 ml

Table 3.1 PBMEC minus Fibronectin medium

3.1.2.2 C6 medium

C6 medium was used for carrying out the transport experiment except one experiment, where PBMEC minus Fib-medium was used and another one, where C6 medium was prepared without serum. In the Table 3.2 below, exact volumes of all compounds are listed. The handling with the medium was always under sterile conditions.

C6 medium	For 1000 ml
Ham´s F12	439.00 ml
IMDM	439.00 ml
200 mM L-glutamine	35.00 ml
10 mg/ml humane Holo-transferrin	500.00 µl
1000 U/ml Heparin	500.00 µl
NCS	75.00 ml
10 000U/ml Penicillin; 10 mg/ml Streptomycin	10.00 ml
250 µg/ml Amphotericin B	1.00 ml

Table 3.2 C6 medium

3.1.2.3 C6 medium without serum

This medium was prepared and used only during one transport experiment to investigate the ability of different substances to bind to the plasma proteins and to see the effect on their ability to cross the BBB when the free fraction of the substances was higher. The only difference to the usual C6 medium is that no NCS was added. Exact volume compositions of all solutions are in the Table 3.3.

C6 medium without serum	For 260 ml
Ham´s F12	124.00 ml
IMDM	124.00 ml
200 mM L-glutamine	9.10 ml
10 mg/ml humane Holo-transferrin	130.00 µl
1000 U/ml Heparin	130.00 µl
10 000U/ml Penicillin; 10 mg/ml Streptomycin	2.60 ml
250 µg/ml Amphotericin B	260.00 µl

Table 3.3 C6 medium without serum

3.1.2.4 Cryo medium

This medium was used only for freezing the cells to store them at - 80 °C or liquid N_2. For this purpose, sterile DMSO was mixed with sterile NCS to get a final concentration of 7 % (v/v) DMSO.

3.1.3 Trypsin/EDTA solution

This solution was used for detaching the cells from their growth surface area during splitting. It was stored sterile. Composition of the solution is described in the Table 3.4

Trypsin/EDTA solution	**For 200 ml**
10x Trypsin/EDTA solution	20.00 ml
10x HBSS	20.00 ml
sterile PBS	154.00 ml
1 M Hepes	2.00 ml
7.5 % (v/v) bicarbonate	2.00 ml
10 000 U/ml Penicillin. 10 mg/ml Streptomycin	2.00 ml

Table 3.4 Trypsin/EDTA solution

3.1.4 Coating

Sterilin disposable pipettes; 10 ml, 25 ml	Berlworld Scientific ldt, Stone, Staffordshire, ST05A, UK
Falcon Tissue Culture Plate, 6 well, flat bottom with low evaporation lid, sterile	Becton Dickinson Austria GesmbH, Am Concorde Park E1/7, A-2320 Schwechat
Falcon Cell Culture Insert, 6 well format, 1 µm poresize, sterile	Becton Dickinson Austria GesmbH, Am Concorde Park E1/7, A-2320 Schwechat
 150703 Collagen bovine 3 mg/ml	MP Biomedicals, Inc. 29525 Fountain Pkwy, Solon, Ohio, 44139

Table 3.5 Material used for coating

Solution:

Collagen solution 0.14 mg/ml	7.6 ml PBS 362 µl Collagen bovine Syringe (100 ml) Cannule for injection Spritzenfilter sterile 0.22 µm 2 blue tubes (50 ml)

Table 3.6 Composition of collagen solution

3.1.5 Cell media

IMDM, Ham´s F12, L(+) Glutamine, NBS, Trypsin/EDTA, Penicillin/Streptomycin, HEPES buffer, PBS buffer, all sterile	Gibco BRL Life Technologies, now invitrogen L.T., Hirschstettengasse 44/1, A-1220 Vienna
51550 Heparin Sodium salt from porcine intestinal mucosa, 250 mg	Fluka BioChemica
T0665 holo-Transferrin human	SIGMA-ALDRICH Handles GmbH, Hebbelplatz 7, A-1100 Vienna
DMSO p.a.	Merck GmbH, Zimbagasse, A-1147 Vienna
A2942 Amphotericin B solution, 50 ml	SIGMA-ALDRICH Handles GmbH, Hebbelplatz 7, A-1100 Vienna

Table 3.7 Cell media

3.1.6 RP-HPLC

DGV-20A5/Prominence Degaser	Shimadzu
SIL – 20AC/Prominence Auto Sampler	Shimadzu
CTO – 20AC/Prominence Column Oven	Shimadzu
SPD – 20A/Prominence UV detector	Shimadzu
RID – 10A/Shimadzu Refractive Index Detector	Shimadzu
CMB – 20A/Prominence Comunications Bus Module	Shimadzu
820975-906 Norrowbore HPLC Column 2.1 x 50 mm packed with Zorbax SB-C8 5 µm	INULA Instrumentelle Analytik, Löwenburggasse 2, A-1082 Wien
821125-915 HPLC Column 2.1 x 12.5 mm packed with Zorbax SB-C8 5 µm	INULA Instrumentelle Analytik, Löwenburggasse 2, A-1082 Wien
Lichrospher column 100 RP-18, 250 x 4 mm, 5 µm pore size LichroCART 4-4 precolumns	Merck KGaA (Darmstadt, Germany

Table 3.8 RP-HPLC components

3.1.7 Substances

Celecoxib	a kind gift of Dr. Maierhofer (AGES PharmMed, Vienna, Austria)
Diclofenac	a kind gift of Dr. Maierhofer (AGES PharmMed, Vienna, Austria)
Ibuprofen	I1892, Sigma-Aldrich, Vienna, Austria
Lornoxicam	a kind gift of Dr. Maierhofer (AGES PharmMed, Vienna, Austria)
Meloxicam	M3935, Sigma-Aldrich, Vienna, Austria
Piroxicam	P5654, Sigma-Aldrich, Vienna, Austria
Tenoxicam	T0909, Sigma-Aldrich, Vienna, Austria
Diazepam	a kind gift of Dr. Maierhofer (AGES PharmMed, Vienna, Austria)
5(6)-Carboxyfluorescein	21877, Fluka, Buchs, Switzerland
Probenecid	a kind gift of Dr. Maierhofer (AGES PharmMed, Vienna, Austria)
Verapamil	a kind gift of Dr. Maierhofer (AGES PharmMed, Vienna, Austria)

Table 3.9 List of substances used for experiments

3.1.8 Others

Methanol	8404, HPLC-analyzed, Malinchrodt Baker B.V., Deventer, Holland
Acetonitril	8257, Malinchrodt Baker B.V., Deventer, Holland
Sterilin disposable pipettes: 10 ml and 25 ml	Barloworld Scientific Limited, Staffordshire, UK
Eppendorf Research pipettes	Eppendorf AG, Hamburg, Germany
CellStar sterile PP-test tubes, 15 ml	Greiner Bio-One GmbH, Germany
CellStar sterile PP-test tubes, 50 ml	Greiner Bio-One GmbH, Germany
Vortex centrifuge	Scientfic Industries Inc., Bohemia, NY, USA

Table 3.10 Other chemicals and equipment used during studies.

3.2 Methods

3.2.1 Treatment of the cell culture`s flask

3.2.1.1 Coating of T25 flask

2 ml of 1 % sterile gelatine solution were added in a new sterile T25 flask to cover the whole surface area equally. The flask was incubated at least for 20 minutes at room temperature. After the gelatine solution was removed, the flask was ready for cell seeding. The coating was done in the laminar flow cabinet to ensure the sterility.

3.2.1.2 Cell subcultivation

If the cell layer was confluent in the T25 flask, the cells were ready for subcultivation (splitting). All solutions used for the splitting were at first pre-warmed at 37 °C in a warming chamber to reach the needed temperature and thereby to prevent the cells from a temperature shock. The old growth medium was removed from the flask and the surface was washed with 2 ml sterile PBS twice. After that, 2 ml of Trypsin/EDTA solution were added and the flask was incubated for 3 – 5 minutes while morphology was observed microscopically. When the cells were spherical, solution was removed. The flask was knocked onto the table to detach the cells from the growth surface. 5 ml of new growth medium were added, the surface was washed at 9 points to detach all cells and the cell suspension was homogenised by pulling up and pushing down the medium at least for 20 times. Required volume of the cell suspension was transferred into a new, gelatine coated T25 flask, usually in a ratio 1:10 or 1:20. The volume in the new flask was completed with growth medium to reach the total volume of 5 ml. The flask was shaken gently to disperse the cells in the whole area. The labelled flask was placed into the incubator for cultivation at 37 °C, 95 % air/5 % CO_2 and 96 % humidity.

3.2.1.3 Change of the growth medium

For the first time the medium was changed the day after cell splitting. Since that, the medium was changed every second day until the cell layer was confluent again and it was needed to split the cells. Before every medium change, the medium was pre-warmed by 37 °C in a warming cabinet. In the laminar air flow cabinet the old medium was removed from the flask and 5 ml of fresh growth medium were added. The flask was replaced into the incubator.

3.2.1.4 Cell freezing

The cells could be frozen in a T25 flask directly, but only for a short period (max. 4 months). In this case, the growth medium was removed and the flask was filled with 2 ml of cold cryo-medium. The flask was given into a fridge (4 °C) for 15 minutes and then placed into a -80 °C freezer.

If the cells should be stored for more than 4 months, the process must be different. At first, the growth medium was removed from the T25 flask with confluent cell layer. The surface was washed twice with 2 ml of sterile PBS and after that 2 ml of Trypsin/EDTA solution were added. The flask was incubated for a couple of minutes (3-5) while morphology was observed under the microscope. When the cells were spherical, solution was removed. The flask was knocked onto the table to detach the cells from the growth surface. After 6 ml of cryo medium (4 °C) were added, the cells were resuspended and homogenised. The suspension was filled as 1.5 ml portions in each cryovial. At first, the cryovials were stored in a fridge (4 °C) for 15 minutes, then replaced into -80 °C freezer, where they stayed overnight and finally given into a liquid nitrogen filled dewar.

3.2.1.5 Cell thawing

The flask was taken out from the -80 °C freezer and incubated at 37 °C until the medium was removable. The medium was removed and 5 ml of fresh pre-warmed growth medium (37 °C) were added. Next day the cells were splitted.

If the cells were stored for longer time, they were in cryovials placed in liquid nitrogen. From there the cryovials were transferred into an incubator (37 °C). When the

cell suspension was removable, the whole content was removed in a new with gelatine coated T25 flask filled with 5 ml of pre-warmed growth medium. Next day the medium had to be changed.

3.2.2 Transwell model

The transwell model consisting of a 6-well plate with six according inserts was used for cell cultivation for experiments. The inserts were placed into the chambers of the 6-well plate and represented the donor chamber of the substances. The plate with six chambers represented the acceptor compartment of the substances (Figure 3.2).

The inserts had on the bottom a semi-permeable membrane with 1 µm pore size and with a total growth area of 4.2 cm^2.

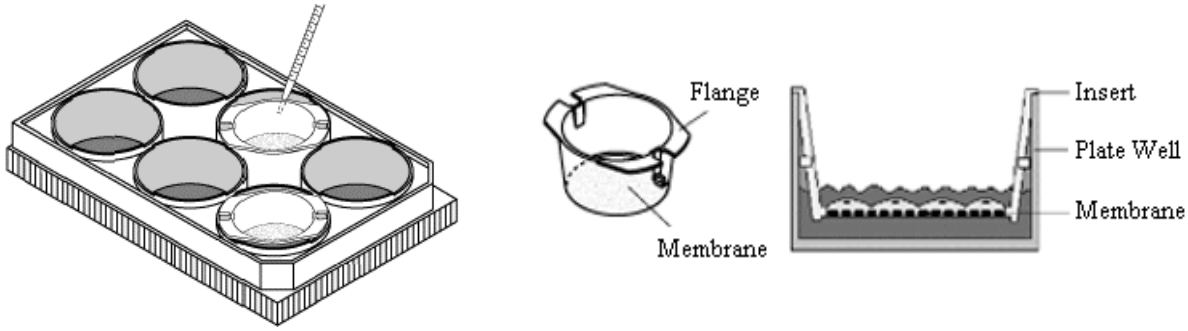


Figure 3.2 Transwell model
Left is the transwell model with six chambers. Right is the image of an insert and a view from the side. Source (Beckton and Dickinson, 2009)

3.2.2.1 Coating of inserts for the transwell model

At first, the inserts were placed into the plate. Both the 6-well plate and the inserts in the original unbroken wrapping were placed into the laminar air flow and opened there. Every insert was touched via tweezers, which was sterilized in a flame before. After replacing two or three inserts, the tweezers were sterilized again.

When all inserts were already in the 6-well plate, the coating could follow. The inserts were coated with 150 µl of 0.14 mg/ml collagen, which was dissolved in sterile PBS. Every portion of collagen was added to each insert with a pipette using a new sterile tip. Finally, the plate was shaken gently to cover the whole surface of the inserts.

Then, the plate was incubated for 1 hour by 37 °C. After one hour the supernatants of collagen were removed and the plate was ready for cell seeding.

3.2.2.2 Cell seeding

The required amount of cells for the experiments was 80 000 cells/cm^2. The exact amount of cells, which had to be given into the inserts, had to be determined via cell counting. 20 µl of cell suspension per counting chamber were added into a Thoma chamber slide and the total number per chamber was established. The average of both per chamber was multiplied by 10^4 to obtain the amount in cells/ml.

The confluent cells from a T25 flask were used for the seeding. The medium was removed from the flask, the surface was washed twice with 2 ml of PBS and then 2 ml of Trypsin/EDTA solution were added. When the cells were spherical, the solution was removed. The flask was knocked onto the table to detach the cells from their growth area. 5 ml of fresh growth medium were added and the cell suspension was homogenised and transferred into a test tube. From this suspension 20 µl were added into the Thoma chamber for counting the cells under the microscope.

Every well of the 6-well plate was filled with 3 ml of pre-warmed growth medium and inserts were filled only with 1 ml. Only in three inserts the precalculated amounts of cell suspension was added (usually less than 1 ml). The other three inserts were used as a blind control. There were treated in the same way as the inserts with cells all the time. When the required cell amount was added into the three inserts for cell cultivating, all inserts were filled with growth medium to the total volume of 2 ml. The plate was shaken gently and placed in an incubator. The cells grew there for 14 days until they were tight enough for an experiment.

Every plate was always labelled with the name of the cell line, passage and generation of the cells, date and short cut of my name (ECV304, Pxx, Gyy, date, IN). It was also important to differ somehow the inserts with cells from that without cells. The conditions for growing in the incubator were 37 °C, 5 % CO_2 and 96 % humidity.

3.2.2.3 Medium change in the transwell model

Usually, the medium was changed immediately the next day after the cell seeding and since that every second or third day. As mentioned before, the blind inserts and the inserts with cells were treated in the same way, what includes the medium changing as well.

The 6-well plate was taken out of the incubator and the cells were checked under the microscope. Then the plate was placed into the laminar air flow. The growth medium was pre-warmed by 37 °C at least for 30 minutes before the medium change. The inserts were emptied directly into to waste bottle by using a tweezers sterilised in the flame before. Empty inserts were placed into another empty sterile plate just to stay there until the medium was changed also in the chambers. After emptying of two or three inserts the tweezers was sterilized again. When all inserts were empty, the medium from the wells was aspirated by using a pipette boy and removed into the waste bottle. Next, each well was filled with 3 ml fresh medium again. Then, the inserts were replaced from the empty plate back into the original plate with fresh medium in the chambers. This replacement was made again by using the sterilised tweezers. If the inserts were back in the original 6-well plate, they were filled with 2 ml of fresh medium as well. The medium was added were carefully, not directly on the cells, but along the insert wall. Finally, the plate was placed back into the incubator, if the TEER measurement did not follow.

3.2.2.4 Measurement of the Transendothelial/Epithelial Electrical Resistance (TEER)

TEER is a significant characteristic of the integrity of the cell layer. The higher the TEER was the tighter the cell monolayer was and the more the permeation was restricted even for ions. The values of TEER were observed starting at day 7 every second day and they were rising up until the day of the experiment (day 14). The final TEER value could influence the process of the experiment itself.

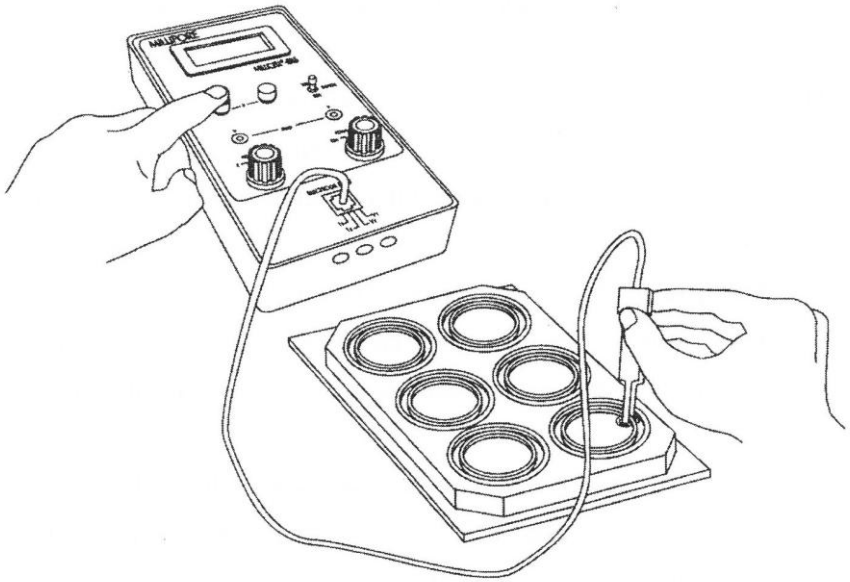


Figure 3.3 The scheme of the apparatus for the TEER measurement.

TEER was measured via a Millipore Millicell Electrical Resistance System (ERS, Millipore Vienna). The apparatus consists of two electrodes, where one of them is longer than the other one. Usually, the electrodes were stored in a test tube with 70 % (v/v) ethanol and before every measurement they were replaced into a test tube with the same growth medium used for cell cultivating for at least 30 minutes. Before every measurement the medium in the 6-well plate was changed and the plate was incubated by room temperature for 1 hour (because the electrical resistance is temperature dependent). While the measurement, the electrodes were placed into the insert (shorter part) and the well (longer part). After the resistance was measured in the three blank chambers, the electrodes were put into ethanol for app. 10 seconds and then immediately into the growth medium again. Then, the measurement followed by the inserts with cells (Figure 3.3). The values from the blank inserts were used for calculation of the real resistance of the cell monolayer. The resistance values were multiplied by surface area to get the TEER values in Ohm*cm^2 (see the formula below).

$$\text{TEER } [\Omega * \text{cm}^2] = (\text{TEER}_{\text{cell}} - \text{average TEER}_{\text{blank}}) * \text{surface area } (4.2 \text{ cm}^2)$$

3.2.3 Transport studies

3.2.3.1 General procedures by the transport experiments

On the day 14 experiments were carried out. Before the experiment was started, the medium in the 6-well plate was changed, the plate was incubated by room temperature for 1 hour and then, TEER was measured. The last TEER measurement was important to confirm the usability of the cells for the experiment.

Although the cells were cultivated in PBMEC minus Fib medium, usually for the experiments C6 medium was used if not mentioned otherwise.

Six clean non-sterile 6-well plates were filled with 3 ml of C6 medium and were put into incubator (37 °C) for one hour. After the TEER was determined, the inserts were emptied directly into the waste bottle by using tweezers and the plate was taken out of the laminar flow box. The following steps of the experiment were not made under sterile conditions anymore. The six plates with pre-warmed medium were taken out of the incubator. Every empty insert was placed into one of these 6 plates so that every insert was in one plate. All plates were labelled to recognise which inserts were in which plates. Next, the inserts were filled with 2 ml of experimental solution, which was prepared before by using also C6 medium (if not mentioned otherwise) as vehicle of the substances. This solution was also preheated at 37 °C before. The filling of the inserts followed always in the same order, started by filling the blank inserts (1 till 3) and thereafter filling the inserts with cells. When the first blank insert was filled, the time started to be measured. An interval of 30 seconds was always kept between filling each insert. It was very important to follow up the exact time, every time variance was noted.

When all inserts were filled with experimental solution, the plates were carefully placed back into the incubator, where they stayed until the experiment was finished.

The inserts were transferred via tweezers in the next well every 40 minutes, overall 6 times and the whole experiment took 4 hours. The order of replacing was always in the same direction and it was symbolized on the lid of the plate to avoid mistakes.

Shortly before the end of the experiment, all plates were taken out of the incubator. In the exact time order, the inserts were taken out of the plates and were put into an empty plate. Immediately the supernatants from the inserts were aspirated and

transferred into eppendorf vials. With collecting the last supernatant from the insert the experiment was finished and the time was not observed any more. Also from all chambers from all six plates samples of the medium with migrated substances were collected into eppendorf vials. All vials were labelled corresponding to the transferring order of the inserts (42 samples). Finally, a sample of pure C6 medium and of experimental stock solution was collected as well. Before any other use, samples were stored in a fridge (4 °C).

3.2.3.2 Internal standards

To normalize the permeability data of NSAIDs internal standards diazepam and carboxyfluorescein (CF) were used to minimise the influence of cell layer's variabilities.

Diazepam is a very lipophilic substance, which can pass transcellularly the BBB via passive diffusion. Diazepam was dissolved in methanol. The sterile filtered stock solution was 10 mM. This solution was added into the experimental solutions every time.

Carboxyfluorescein was reported as a fluorescent marker for the paracellular transport route. It was dissolved in water to get a concentration of 400 µM but not sterile filtered. This solution was added also to test solutions except one group study to investigate the influence of the CF on the transport abilities of the substances. The chemical structure and molecular weight is shown in the Table 3.11.

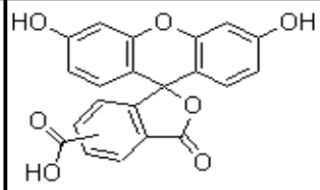
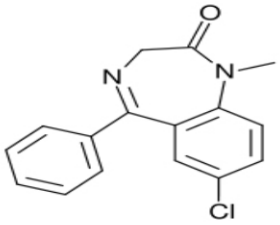
Substance	Structure	MW [g/mol]	Half life [hours]
Carboxyfluorescein		376.32	-
Diazepam		284.74	30 - 80

Table 3.11 CF and diazepam used as internal standards
Source (Lincová, Farghali et al., 2007)

3.2.3.3 Stock solutions with test substances

Tested substances were dissolved either in water (Aqua purificata) or in DMSO, which differed from their physical-chemical characteristics. Diclofenac was dissolved in water and the final concentration of the stock solution was 3 mM. Ibuprofen and meloxicam were also dissolved in water and the final concentration of each solution was 10 mM. Tenoxicam and piroxicam were dissolved in DMSO and the concentration of each solution was 10 mM. There was prepared another solution of tenoxicam and piroxicam, where the substances were mixed together in DMSO and the final concentration was 100 mM. This solution was used for the group studies. Two solutions of celecoxib dissolved in DMSO were prepared. One with a concentration of 10 mM, which was used for single study, and another one with concentration of 100 mM used for group studies. All water solutions were sterile filtered.

These stock solutions were used for preparing the test solutions as necessary. The test solutions contained always 100 μM of the respective NSAIDs and additionally 1 % DMSO (except one group study with 0.1 % DMSO to check the influence of DMSO). As internal standards diazepam (100 μM) and carboxyfluorescein (5 μM) were also added to the solutions (except one group study without CF). The vehicle of the substances in the test solution was usually C6 medium (if not mentioned otherwise). The solutions were prepared under sterile conditions.

3.2.3.4 Specific blockers used for transport group studies

During two group studies specific transporter blockers were used.

In one study probenecid was added. Probenecid is used for the treatment of gout. In cell biology probenecid is a useful blocker for the transport of organic anions by OATs or MRP transporter systems. Probenecid as an organic acid may compete with other organic acids and if it binds to an appropriate transporter it makes it unavailable for other substances.

In another study verapamil was used. It is an L-type calcium channel blocker. By inhibiting the voltage dependent Ca^{2+} channel, it leads to smooth muscles relaxation and though it causes negative inotropic and chronotropic effects on the heart. On the other hand, verapamil can inhibit also efflux mechanism of P-glycoprotein. Verapamil is

usually used for the treatment of angina pectoris, hypertension and cardiac arrhythmia. It can also be used as a vasodilatator generally and by regular use it protects from chest pain. It was also reported that verapamil could be used as an alternative medicine instead of valproic acid or lithium for treatment of mania and hypomania in pregnant patients (Giannini et al., 1984; Giannini et al., 2000).

Probenecid as well as verapamil was dissolved in DMSO to reach a final concentration of 100 mM of the stock solutions.

The Table 3.12 shows chemical structures, molecular weights and plasma half lives of probenecid and verapamil.

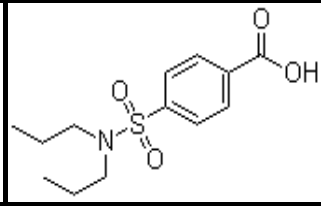
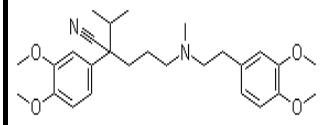
Substance	Structure	MW [g/mol]	Half life [hours]
Probenecid		285.36	3.5 – 12
Verapamil		491.06	2 – 7.5

Table 3.12 Data of probenecid and verapamil
Chemical structure, molecular weight and plasma half lives of verapamil and probenecid used in the studies as specific blockers of transport mechanisms.

3.2.3.5 Fluorescence measurement

To obtain the permeability of CF, the fluorescence of the samples were measured by a microplate reader (BMG, excitation: 485 nm, emission: 520-535 nm).

Three times 100 µl of each sample were pipetted into the fluorescence plate at room temperature and were measured. Also the stock solution of mixed substances and the pure medium used for the experiment were analysed.

The fluorescence data were used for determination of the clearance. The calculation and determination of PS and PE values for CF was the same as for single substances and diazepam determined by RP-HPLC, which will be mentioned in section 3.2.3.7.1.

3.2.3.6 Reversed-phase high performance liquid chromatography (RP-HPLC)

The parts of the RP-HPLC apparatus (pump, autosampler, detector, column thermostat, columns) are listed in section 3.1.6.

Acetonitril (ACN) or methanol (MeOH) mixed with potassium phosphate buffer pH = 2.5 (except single study with celecoxib, where the pH was 3.5) were used as eluents. The eluents were degassed by ultrasonic treatment for at least 20 minutes before use. The used ratio of ACN:buffer or MeOH:buffer was tested before each measurement. A mixture 50:50 of acetonitril and water was used for washing the injection needle. Every sample was measured three times and the average of the obtained peak areas was used for the calculation of the permeability coefficients.

Before HPLC analysis, the samples had to be purified. 500 µl of each sample were transferred into a new eppendorf vial. 500 µl of acetonitril or methanol were added (dependent on which solvent was used as eluent) and vortexed. Samples stayed at 4 °C at least for 30-60 minutes to support the process of precipitation. After that, the samples were centrifuged at 12 000 rpm for 10 minutes and supernatants were collected into HPLC-vials. Then, samples were ready for analysis and until that time, they were stored at 4 °C. Table 3.13 summarises the conditions of the HPLC-methods for each single transport experiment.

		RP-HPLC methods							
No	**Datum**	**Buffer pH**	**Buffer [%]**	**ACN [%]**	**MeOH [%]**	**Time [min]**	**t [°C]**	**Flow rate [ml/min]**	**Type of study**
1	13/11/2008	2.5	40	60	-	10	25	1	single
2	20/11/2008	2.5	40	60	-	10	25	1	single
3	27/11/2008	2.5	53	47	-	13	25	1	single
4	4/12/2008	2.5	53	47	-	13	25	1	single
5	11/12/2008	2.5	42	58	-	10	25	1	single
6	18/12/2008	2.5	46	54	-	10	25	1	single
7	3/2/2009	2.5	56	44	-	15	25	1	group
8	10/2/2009	2.5	56	44	-	15	25	1	group
9	17/2/2009	2.5	40	-	60	18	25	1.3	group
10	24/2/2009	3.5	35	-	65	15	25	1	single
11	3/3/2009	2.5	40	-	60	15	25	1.5	group
12	10/3/2009	2.5	40	-	60	16	25	1.3	group
13	17/3/2009	2.5	40	-	60	16	25	1.3	group
14	24/3/2009	2.5	40	-	60	18	25	1.3	group
15	31/3/2009	2.5	40	-	60	18	25	1.3	group
16	21/4/2009	2.5	40	-	60	18	25	1.3	group

Table 3.13 represents the conditions of RP-HPLC measurement by every study.

Ultraviolet (UV) detection of the RP-HPLC was set up to three wavelengths – 220, 254 and 370 nm. Diazepam, ibuprofen and diclofenac were detected at 220 nm during single and group studies. Celecoxib was detected at 254 nm during single study (Zarghi et al., 2006) and during the group studies at 220 nm. Tenoxicam, meloxicam and piroxicam were detected at 370 nm during both types of study. Exact composition of eluents and further conditions of the analysis are shown in the Table 3.13. Injection volume was changing in relation to the used column. While using the column of length 250 x 4 mm (Lichrospher), the injection volume was 50 µl; except analysing samples from single study with celecoxib, where the injection volume was 100 µl (to allow better detection of celecoxib peaks). When the used column was only 150 x 4 mm long (Zorbax), the injection volume was decreased to 20 µl. The time of the analyses varied from 10 to 20 minutes. Normally, the Lichrospher column was used for the single substance studies, whereas the Zorbax column was applied for group substance studies. The pre-column of the Zorbax column was changed after an increase of app. 10-20 bar of the pressure in order to ensure long lifetimes of the column.

3.2.3.7 Single studies – conditions, calculations

At first, single studies for each substance were carried out to investigate their general permeation abilities to cross the used BBB *in vitro* model.

Each solution consisted of 100 µM substance of the according NSAID, 100 µM diazepam, 5 µM CF, 1 % DMSO in C6 medium (Table 3.14). Used passages and generations of the cell line are also mentioned in the Table 3.14. Generation means how many passages the cells had been cultivated in minus Fib Medium.

No	Datum	ECV passage	Generation	Study	Composition of the solution
1	13/11/2008	152	11	single	100 µM **Diclofenac** 100 µM Diazepam, 5 µM CF 1 % DMSO in C6 medium
2	20/11/2008	153	12	single	100 µM **Ibuprofen** 100 µM Diazepam, 5 µM CF 1 % DMSO in C6 medium
3	27/11/2008	154	13	single	100 µM **Piroxicam** 100 µM Diazepam, 5 µM CF 0.98 % DMSO in C6 medium
4	4/12/2008	155	14	single	100 µM **Tenoxicam** 100 µM Diazepam, 5 µM CF 1 % DMSO in C6 medium
5	11/12/2008	156	15	single	100 µM **Meloxicam** 100 µM Diazepam, 5 µM CF 1 % DMSO in C6 medium
6	18/12/2008	157	16	single	100 µM **Lornoxicam** 100 µM Diazepam, 5 µM CF 1 % DMSO in C6 medium
7	24/2/2009	164	23	single	100 µM **Celecoxib** 100 µM Diazepam, 5 µM CF 1 % DMSO in C6 medium

Table 3.14 Overview of experimental conditions
The table describes every single study including the passage and generation of the used cell line and further components of the experimental solutions. During the preparation of the test solution with piroxicam a mistake occurred and the final amount of DMSO was only 0.98 %.

3.2.3.7.1 Calculation of the permeability coefficient (PE)

During the experiment, the inserts as donors of the substances were transferred at certain time intervals into following wells with medium only, which represented the acceptor chamber. The calculation of the permeability coefficients is based on the clearance principle. At first, the clearance had to be calculated.

In pharmacokinetics clearance [µl/min] is a parameter, which determines the amount of biological fluids (plasma or blood) totally cleared from a substance during a certain time.

In the following described calculation, the cleared volume [µl] only is termed as clearance. Although this is maybe contradictory to the classical definition of the clearance, it is routinely used like this in the BBB-field and in our group's practice. For the purpose to calculate the clearance, the following equation was used (1) (Neuhaus et al., 2006).

(1) $$\text{Clearance [µl]} = (C_A * V_A)/C_D$$

C_A... concentration in acceptor chamber

V_A...total volume in acceptor chamber

C_D...concentration in donor insert

By using integrated peak areas, the calculation was a little different. The peak area for a certain time point ($Peakarea_t$) - 40 to 240 minutes - was multiplied by the total volume in the well (3 ml = 3000 µl) and divided by peak area of the substance in the stock solution ($3000/Peakarea_{STL}$) (2).

(2) $$\text{Clearance [µl]} = Peakarea_t * (3000/Peakarea_{STL})$$

By replacing the inserts in time, the concentration in the donor insert was sinking equally to the number of transferring steps. To take this into account, by any further time point was from the $Peakarea_{STL}$ subtracted the summation of the peak areas from the time points before and multiplied by 1.5, which is the ratio between the two different volumes in the well and in the insert (3000/2000 = 1.5) (3).

(3) $$\text{Clearance [µl]} = Peakarea_t * (3000/Peakarea_{STL} - \sum Peakarea_{t-1} * 1.5)$$

To get the final clearance value, all clearances by different time points were summed up. Table 3.15 shows an example for the clearance calculation of piroxicam.

Calculation of clearance for piroxicam

Time [min]	0	40	80	120	160	200	240	240
Cell-Well CA								
MW Peakarea		58985.67	58021.67	60055.00	62501.33	49715.0	61941.7	1114802.7
Clearance		108.34	112.67	123.59	137.09	117.07	154.93	3022.51
Clearance Sum	0	108.34	221.02	344.60	481.69	598.76	753.69	3776.21

Time [min]	0	40	80	120	160	200	240	240
Cell-Well CB								
MW Peakarea		49386.67	57442.67	68036.00	64402.00	51738.0	55482.0	1161880.3
Clearance		90.71	110.52	138.56	140.92	121.79	139.07	3130.07
Clearance Sum	0	90.71	201.23	339.79	480.71	602.50	741.57	3871.64

Time [min]	0	40	80	120	160	200	240	240
Cell-Well CC								
MW Peakarea		48279.33	61689.67	64314.33	59753.33	50279.0	50640.7	1166178.0
Clearance		88.68	118.56	131.40	130.67	117.63	125.88	3093.60
Clearance Sum	0	88.68	207.24	338.64	469.31	586.94	712.82	3806.42

Time [min]	0	40	80	120	160	200	240	240
Cell-Well BA								
MW Peakarea		199626.3	154827.6	112474.0	96310.3	96797.6	63611.0	792307.67
Clearance		366.66	348.22	306.29	309.70	368.30	296.66	4338.57
Clearance Sum	0	366.66	714.88	1021.17	1330.87	1699.17	1995.82	6334.40

Time [min]	0	40.0333	80	120	160	200	240	240
Cell-Well BB								
MW Peakarea		192617.3	195330.0	116829.6	109364.0	70208.6	74434.3	767993.33
Clearance		353.79	435.87	333.35	374.46	295.77	368.00	4653.07
Clearance Sum	0	353.79	789.66	1123.01	1497.48	1793.25	2161.25	6814.32

Time [min]	0	40	80	120	160	200	240	240
Cell-Well BC								
MW Peakarea		184785.6	149031.3	120677.6	112724.0	98721.0	65481.0	827443.33
Clearance		339.40	329.68	319.65	355.38	378.48	309.64	4629.50
Clearance Sum	0	339.40	669.08	988.73	1344.11	1722.59	2032.23	6661.73

Table 3.15 Example of calculation of clearance

The table shows clearance for every well (CA to CC are three inserts with cell monolayer, BA to BC are three insert without cells - blanks) in every certain time point. The peak areas were determined from chromatograms after analysing the samples by RP-HPLC; it is the average from three values. Clearance is determined for any certain time point. The final clearance is expressed by the sum, which shows an increasing trend.

As shown in the graph (Figure 3.4 - for piroxicam single study) there were six different lines representing the increasing clearance in relation to the time. They represent the three blank inserts (BA, BB, BC) and three inserts with cell monolayer (CA, CB, CC). According to this data, the slope was calculated after linear regression analysis by using Microsoft Excel program. Based on the obtained slopes, the PS values were determined.

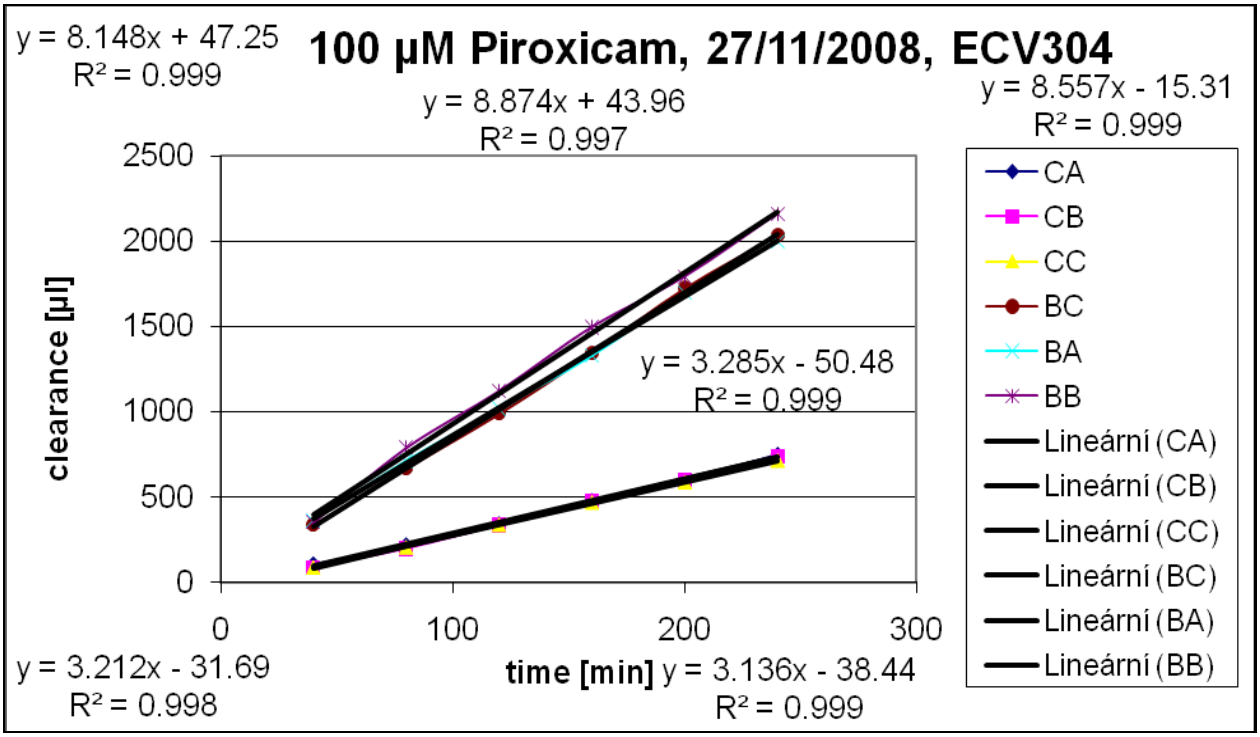


Figure 3.4 Data of piroxicam shown in a graph
Example of a graph showing the dependence of clearance on the time interval 40-240 minutes.

The PS value represented the relation between the permeation and the surface (PS = permeability * surface). Of course there were two different PS values. PS-blind values were calculated for blanks and PS_{all} were calculated for cell inserts. PS_{all} represented the permeability restricted by both cell monolayer and membrane of the insert. To get the permeability of the cell monolayer only, these two reciprocal values were subtracted ($1/PS_{cell} = 1/PS_{all} - 1/PS_{blank}$). $1/PS_{cell}$ was converted into PS_{cell} ($1/\ 1/PS_{cell}$). These values could be finally converted into the corresponding permeability coefficients PE [µm/min]. Then, the PS_{cell} was divided by the surface area of the insert (4.2 cm^2) and divided by 1000 to get the dimension in [cm/min]. By multiplying this value with 10 000 PE in [µm/min] was obtained.

	PS-blindvalue	PS-all	1/PS-cell	PS-cell	PE [cm/min]	PE [µm/min]	PE [cm/s]
Cell 1	8.1488	3.2122	0.18860	5.30235	0.00126	12.62464	0.00002104
Cell 2	8.8743	3.285	0.19173	5.21569	0.00124	12.41832	0.00002069
Cell 3	8.5572	3.1361	0.20201	4.95033	0.00118	11.78650	0.00001964
Cell average	**8.526766667**	**3.2111**	**0.19414**	**5.15087**	**0.00123**	**12.26397**	**0.00002044**
St. deviation	0.363706205	0.074456					
	8.526766667	3.2122	0.19404	5.15370	0.00123	12.27071	0.00002045
	8.526766667	3.285	0.18714	5.34370	0.00127	12.72310	0.00002120
	8.526766667	3.1361	0.20159	4.96057	0.00118	11.81089	0.00001968
	8.526766667	**3.2111**	**0.19414**	**5.15087**	**0.00123**	**12.26397**	**0.00002044**
St. dev. N				0.1915658		0.372412	
St. deviation						0.456109	
						12.26823	Average
		3.2122	0.31131	3.21220	0.00076	7.64810	0.00001275
		3.285	0.30441	3.28500	0.00078	7.82143	0.00001304
		3.1361	0.31887	3.13610	0.00075	7.46690	0.00001245
	-	**3.2111**	**0.31153150**	**3.20995**	**0.00076**	**7.64273**	**0.00001274**
St. dev. N						0.144746	
St. deviation						0.177276	
						7.64548	Average

Table 3.16 Example of calculation of PE values by piroxicam single study
The table represents the calculation of the PE according to the graph (Fig.3.4) for time interval 40-240 minutes. PS-blanks and PS-all were obtained from calculation of the slope in the graph. $1/PS_{cell}$ is a reciprocal difference of PS_{all} and PS_{blanks}. PS_{cell} devided by surface area and 1000 represents PE in [cm/min], which is further multiplied by 10000 to get the dimension [µm/min].

The Table 3.16 shows three parts. In the first one, all single PS_{blank} values were used for the calculation of PE. An average was made from the three final PE values (marked with yellow colour on top). In the second (middle) part, an average of PS_{blanks} was used to minimise the variability of permeability by blank inserts. The average of PE was marked with red colour (first one). In the last part, permeability for inserts containing cell monolayer and insert's membrane was determinated. Hence, the average value of PE was lower (second red marked line), which corresponded well with presence of a larger barrier.

Effect of correction:

		EFFECT of Correction [%]	
12.27071	7.64810	160.4414	
12.72310	7.82143	162.6697	
11.81089	7.46690	158.1765	
		160.42921	Average
		2.246641	St. deviation

Table 3.17 Effect of correction for time interval 40-240 minutes.

Effect of correction was derived to demonstrate the influence of the collagen-coated membrane (PS_{blank}). It was the ratio between PE_{cell} and PE_{all} expressed in percent. The lower the percentage was the higher the cell monolayer's participation on the restriction was and the less influence was caused by the insert's membrane itself (Table 3.17).

Determination of clearance was also calculated for the time interval 0-40 minutes. The concentration during first permeation was the highest and by transferring further it was decreased because of the loss of molecules according to amount of passing through the barrier. The higher the concentration was the higher amount of substances has passed the barrier. This fact corresponded to increased slopes and just a lower increase of PE values. Anyway, the determination of permeability coefficients by two different time intervals offered the possibility to observe the time influence on the transport studies. For example, clearance data are demonstrated from the time interval 0-40 minutes in form of a graph here (Figure 3.5) followed by Table 3.18 and 3.19 with the calculation of PE values and determination of the effect of correction.

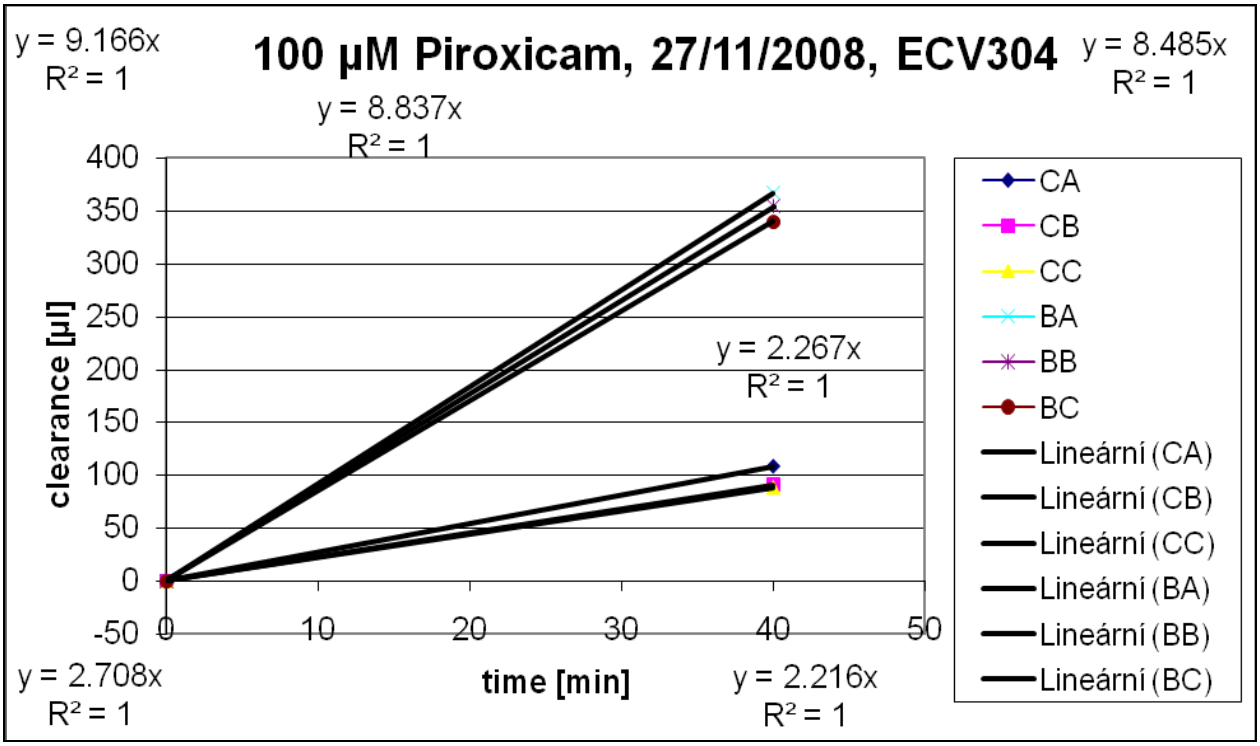


Figure 3.5 Data of piroxicam shown in a graph
Example of a graph showing the dependence of clearance in the time interval 0-40 minutes.

	PS-blindvalue	PS-all	1/PS-cell	PS-cell	PE [cm/min]	PE [µm/min]	PE [cm/s]
Cell 1	9.1665	2.7085	0.26012	3.84445	0.00092	9.15346	0.000015256
Cell 2	8.8373	2.2678	0.32780	3.05065	0.00073	7.26345	0.000012106
Cell 3	8.4851	2.2169	0.33323	3.00096	0.00071	7.14514	0.000011909
Cell average	**8.829633**	**2.39773**	**0.30381**	**3.29158**	**0.00078**	**7.83709**	**0.000013062**
St. deviation	0.340765	0.27033					
	8.829633	2.7085	0.25595	3.90697	0.00093	9.30230	0.000015504
	8.829633	2.2678	0.32770	3.05156	0.00073	7.26562	0.000012109
	8.829633	2.2169	0.33783	2.96011	0.00070	7.04788	0.000011746
	8.829633	**2.39773**	**0.30381**	**3.29158**	**0.00078**	**7.83709**	**0.000013062**
St. dev. N				0.52227		1.01532	
St. deviation						1.243508	
						7.87193	Average
		2.7085	0.36921	2.70850	0.00064	6.44881	0.000010748
		2.2678	0.44096	2.26780	0.00054	5.39952	0.000008999
		2.2169	0.45108	2.21690	0.00053	5.27833	0.000008797
	-	**2.39773**	**0.42042**	**2.37860**	**0.00057**	**5.66334**	**0.000009439**
St. dev. N						0.525537	
St. deviation						0.643649	
						5.70889	Average

Table 3.18 Determination of PE values demonstrated on piroxicam single study
Description more in detail was demonstrated by Table 3.16.

Effect of correction:

		EFFECT of Correction [%]	
9.30230	6.44881	144.24	
7.26562	5.39952	134.56	
7.04788	5.27833	133.52	
		137.44	Average
		5.915	St. deviation

Table 3.19 Effect of correction for time interval 0-40 minutes.

3.2.3.7.2 Ratios

Although the treatment of all 6-well plates during the cell cultivation was always the same with tendency to keep them for the whole time under the same conditions, variations in results occurred.

To minimise the variation evoked either by the collagen-coated membrane or by the cell monolayer, the permeation coefficients were expressed as ratios normalised to the internal standards – diazepam and carboxyfluorescein. Therewith, we got another possibility to compare the obtained data and could confront the accuracy rate of the experiments.

The ratio diazepam/substance expressed the factor how much slower the substance permeated in comparison to diazepam (or also how much faster diazepam than the substance was). For this purpose the PE_{cell} values for diazepam and the substance, calculated from PS_{blanks} average and PS_{cell} for each insert, were used (Table 3.20). In the second case, only PE_{all} data were used (Table 3.21). The final average and the standard deviation were determinated. Because PE values were calculated for two different time intervals - 0-40 minutes and 40-240 minutes - also the ratios were calculated for both time intervals.

PE_{cell} ratio diazepam/piroxicam	
2.39	
2.34	
2.48	
2.41	Average
0.073	St. deviation

PE_{all} ratio diazepam/piroxicam	
1.73	
1.70	
1.77	
1.74	Average
0.035	St. deviation

Table 3.20 Ratios
PE_{cell} (left) and PE_{all} (right) ratios of diazepam/substance by time interval 40-240 minutes.

PE_{cell} ratio diazepam/piroxicam	
2.86	
2.58	
2.63	
2.69	Average
0.149	St. deviation

PE_{all} ratio diazepam/piroxicam	
1.86	
1.87	
1.89	
1.87	Average
0.019	St. deviation

Table 3.21 Ratios
PE_{cell} (left) and PE_{all} (right) ratios of diazepam/substance by time interval 0-40 minutes.

The same ratios were calculated for the relationship between CF and the substances. CF was always the slowest substance of all. In this case, the ratio expressed how much slower CF was compared to the substance (Table 3.22 and 3.23).

PE_{cell} Ratio CF/piroxicam		**PE_{all}** Ratio CF/piroxicam	
0.35		0.48	
0.37		0.51	
0.38		0.51	
0.37	Average	0.49	Average
0.012	St. deviation	0.014	St. deviation

Table 3.22 Ratios
PE_{cell} (left) and PE_{all} (right) ratios of CF/substance by time interval 40-240 minutes.

PE_{cell} Ratio CF/piroxicam		**PE_{all}** Ratio CF/piroxicam	
0.31		0.39	
0.44		0.52	
0.54		0.61	
0.43	Average	0.51	Average
0.113	St. deviation	0.109	St. deviation

Table 3.23 Ratios
PE_{cell} (left) and PE_{all} (right) ratios of CF/substance by time interval 0-40 minutes.

Finally, also the ratio between diazepam and CF was observed, describing how much slower CF was compared to diazepam (Table 3.24 and 3.25).

PE_{cell} ratio diazepam/CF		**PE_{all}** ratio diazepam/CF	
6.75		3.59	
6.28		3.36	
6.58		3.51	
6.53	Average	3.48	Average
0.236	St. deviation	0.118	St. deviation

Table 3.24 Ratios
PE_{cell} (left) and PE_{all} (right) ratios of diazepam/CF by time interval 40-240 minutes.

PE_{cell} ratio diazepam/CF		**PE_{all}** ratio diazepam/CF	
9.23		4.69	
5.83		3.58	
4.90		3.09	
6.66	Average	3.79	Average
2.279	St. deviation	0.816	St. deviation

Table 3.25 Ratios
PE_{cell} (left) and PE_{all} (right) ratio of diazepam/CF by time interval 0-40 minutes.

3.2.3.8 Group studies – conditions, calculations

Group studies were made generally in the same way as the single studies. Table 3.26 describes the numbers of passages and generations by used cell line and also compositions of experimental solutions used for each study in detail. The solutions for experiments were mixed according to calculations by using stock solutions.

The calculation and determination of PS and PE values of each substance was exactly the same as mentioned in the description of the single studies (section 3.2.3.7).

Furthermore, ratios between diazepam/substance, substance/CF and diazepam/CF were determined.

Different conditions were chosen to gain new informations, which were then compared to each other. In all group studies lornoxicam was missing because its analysis in the group study was impossible.

The first and the second group study differed in the total amount of DMSO. Generally, 1 % DMSO was present in all studies. One group study with 0.1 % DMSO was made to investigate the influence of DMSO on the transport abilities of NSAIDs.

The third study was carried out in PBMEC minus Fib medium, which was commonly used for cell cultivation.

The fourth study offered a possibility to test the influence of plasma proteins and the binding of substances to them because the experiment was accomplished in serum-free C6 medium.

The study without CF (5) showed the influence of CF on the transport abilities of NSAIDs.

In the study number 6 celecoxib was added additionally in order to incorporate this important COX-2 blocker into the studies.

In the next two following studies (7, 8) two different transport mechanism‘s blockers were added – probenecid and verapamil. For this reason, meloxicam had to be excluded because it overlapped with the blockers in RP-HPLC chromatograms. These two studies differed a bit in the process. In general, in the C6 medium used for the experiment blocker was also added in final concentration 100 µM. The donor chambers were filled and pre-warmed with this medium. In addition, the medium with the blocker was given into the inserts and they were pre-incubated for 30 minutes by 37 °C.

Afterwards the inserts were emptied, they were filled with the experimental solution included chosen NSAIDs, internal standards and specific blocker again. The experiment was started and the following steps were already the same as during other experiments without a blocker.

Finally, the last study (9) was made without meloxicam as well as without any blockers. This offered the possibility to compare these studies and to exclude the influence of the missing meloxicam on the results.

No	Datum	ECV passage	Generation	Study	Composition of the solution
1	3/2/2009	161	20	group	100 µM Diclo, Ibu, Tenox, Melox, Pirox 100 µM Diazepam, 5 µM CF 1 % DMSO in C6 medium
2	10/2/2009	162	21	group	100 µM Diclo, Ibu, Tenox, Melox, Pirox 100 µM Diazepam, 5 µM CF **0.1 % DMSO** in C6 medium
3	17/2/2009	163	22	group	100 µM Diclo, Ibu, Tenox, Melox, Pirox 100 µM Diazepam, 5 µM CF 1 % DMSO in **PBMEC -Fib medium**
4	3/3/2009	165	24	group	100 µM Diclo, Ibu, Tenox, Melox, Pirox 100 µM Diazepam, 5 µM CF 1 % DMSO in **C6 medium without serum**
5	10/3/2009	166	25	group	100 µM Diclo, Ibu, Tenox, Melox, Pirox 100 µM Diazepam, **without 5 µM CF** 1 % DMSO in C6 medium
6	17/3/2009	167	26	group	100 µM **Celec,** Diclo, Ibu, Tenox, Melox, Pirox 100 µM Diazepam, 5 µM CF 1 % DMSO in C6 medium
7	24/3/2009	168	27	group	100 µM Celec, Diclo, Ibu, Tenox, Pirox 100 µM Diazepam, 5 µM CF 1 % DMSO in C6 medium **with 100 µM Probenecid**
8	31/3/2009	169	28	group	100 µM Celec, Diclo, Ibu, Tenox, Pirox 100 µM Diazepam, 5 µM CF 1 % DMSO in C6 medium **with 100 µM Verapamil**
9	21/4/2009	174	33	group	100 µM Celec, Diclo, Ibu, Tenox, Pirox 100 µM Diazepam, 5 µM CF 1 % DMSO in C6 medium

Table 3.26 Exact experimental conditions of group studies

The table shows compositions of test each solution used during the group studies, including the passages and generations of the used cell line.

4 RESULTS

4.1 Single studies

In total seven single studies were carried out. PS and PE values were determined and for a better overview all data were summarised in tables (Table 4.2 until 4.5). It was also necessary to distinguish the two different time intervals.

4.1.1 Time interval 0-40 minutes

Table 4.2 represents data from the time interval 0-40 minutes. Permeability coefficients PE_{cell} were the most interesting values to observe. Obviously, the ranking of substances according to their ability to cross the barrier resulted from the Table 4.2. The ranking of NSAIDs including the internal standards was the following:

diazepam → piroxicam → ibuprofen → tenoxicam → carboxyfluorescein (CF) → meloxicam ⟶ lornoxicam ⟶ diclofenac ⟶ celecoxib

Diazepam was the fastest substance and celecoxib the slowest one.

It is also very interesting that CF was faster than some other substances. It pointed to the fact that these data were from the beginning of the studies, where no transport equilibrium was reached. The ranking was going to change by time as it will be shown further.

Because the PE values from the internal standards diazepam and CF were not equal comparing the different single studies, the ratios were calculated. The normalised data were summarised in the Table 4.3.

If ratios diazepam/substance were observed, the ranking changed to the following:

piroxicam → tenoxicam → ibuprofen → lornoxicam → meloxicam → diclofenac → celecoxib

In this relation the ranking has changed. Ibuprofen switched the positions with tenoxicam and also, lornoxicam switched with meloxicam. However, the numeral values of these two switched pairs were very similar and if we took note of the standard deviations by these values, it would show that they overlapped each other. In this case, we can say that the change in the ranking was not significant.

On the other hand, when we observed the ranking in relation to CF, it also differed.

piroxicam → ibuprofen → tenoxicam → meloxicam → lornoxicam →diclofenac → celecoxib

We can see that the substances pairs ibuprofen – tenoxicam and meloxicam – lornoxicam have replaced their positions again, conversely to the ratios diazepam/substance. Also here holds the same findings as before. The numeral values of the couple ibuprofen – tenoxicam are very similar and if we have a look at the standard deviation, their values overlapped. In case of the pair meloxicam – lornoxicam, the values are not that similar anymore and also the deviations are not overlapping each other's values. Hence, we can say that this ranking is more reliable. Particularly this finding is supported by the ranking of PE_{cell} values itself.

The possible influence of the tightness of the cell layers, reflected in TEER values, on the permeability especially of CF should not be forgotten. The low TEER during the study with tenoxicam may play some role and so influence the position in the ranking (Table 4.1). However, studying the permeability values of the paracellular marker CF no correlation between TEER values and CF-permeability was observed. Thus, it can be assumed that the tightness of the cell layers were quite constant.

Substance	**TEER [Ohm*cm²]**
Tenoxicam	117.6 ± 2.42
Lornoxicam	127.4 ± 2.42
Ibuprofen	128.8 ± 12.83
Celecoxib	128.8 ± 2.42
Piroxicam	133.0 ± 7.27
Diclofenac	134.4 ± 6.42
Meloxicam	144.2 ± 2.42

Table 4.1 TEER values
Average of TEER values measured on the day of experiment.

Single studies/Time interval: 0-40 min.

Date	Substance	PS_{blank} [µl/min]	PS_{all} [µl/min]	PS_{cell} [µl/min]	PE_{all} [µm/min]	PE_{cell} [µm/min]	EoC [%]
27/11/2008	Piroxicam	8.83 ± 0.34	2.40 ± 0.27	3.29 ± 0.52	5.71 ± 0.64	7.87 ± 1.24	137.44 ± 5.92
	Diazepam	9.16 ± 0.28	4.49 ± 0.47	8.82 ± 1.93	10.70 ± 1.12	21.30 ± 4.60	197.69 ± 21.12
	CF	9.52 ± 0.47	1.21 ± 0.14	1.38 ± 0.19	2.87 ± 0.34	3.29 ± 0.45	114.51± 1.99
20/11/2008	Ibuprofen	6.62 ± 0.19	1.53 ± 0.11	1.99 ± 0.20	3.65 ± 0.27	4.75 ± 0.47	130.13 ± 2.96
	Diazepam	7.95 ± 0.91	3.89 ± 0.30	7.61 ± 1.11	9.26 ± 0.71	18.25 ± 2.64	196.38 ± 13.92
	CF	9.46 ± 0.18	1.29 ± 0.06	1.50 ± 0.07	3.07 ± 0.13	3.56 ± 0.18	115.80 ± 0.79
4/12/2008	Tenoxicam	8.78 ± 0.47	1.59 ± 0.07	1.95 ± 0.10	3.79 ± 0.16	4.63 ± 0.24	122.17 ± 1.16
	Diazepam	9.64 ± 0.59	4.10 ± 0.30	7.13 ± 0.94	9.75 ± 0.71	17.05 ± 2.23	174.31 ± 9.72
	CF	10.92 ± 0.57	1.37 ± 0.15	1.57 ± 0.20	3.26 ± 0.37	3.74 ± 0.47	114.37 ± 1.83
11/12/2008	Meloxicam	6.95 ± 0.16	1.24 ± 0.13	1.51 ± 0.19	2.96 ± 0.31	3.61 ± 0.45	121.84 ± 2.71
	Diazepam	8.01 ± 0.12	3.89 ± 0.49	7.58 ± 2.00	9.27 ± 1.17	18.42 ± 4.75	196.59 ± 24.93
	CF	9.08 ± 0.15	1.35 ± 0.32	1.59 ± 0.45	3.22 ± 0.75	3.82 ± 1.06	117.65 ± 4.92
18/12/2008	Lornoxicam	7.83 ± 0.47	1.15 ± 0.12	1.35 ± 0.17	2.73 ± 0.29	3.21 ± 0.41	117.20 ± 2.18
	Diazepam	9.29 ± 0.64	3.86 ± 0.45	6.61 ± 1.38	9.20 ± 1.07	15.92 ± 3.29	171.98 ± 14.89
	CF	9.98 ± 0.95	1.53 ± 0.11	1.81 ± 0.15	3.65 ± 0.25	4.31 ± 0.35	118.12 ± 1.47
13/11/2008	Diclofenac	6.62 ± 0.34	0.89 ± 0.26	1.03 ± 0.36	2.12 ± 0.63	2.48 ± 0.85	115.70 ± 5.38
	Diazepam	7.66 ± 0.51	3.96 ± 0.49	8.21 ± 2.31	9.44 ± 1.17	20.04 ± 5.50	209.84 ± 30.11
	CF	9.29 ± 0.09	1.24 ± 0.26	1.43 ± 0.35	2.94 ± 0.62	3.41 ± 0.83	115.43 ± 3.76
24/2/2009	Celecoxib	8.16 ± 0.12	0.30 ± 0.04	0.32 ± 0.05	0.73 ± 0.11	0.75 ± 0.11	103.89 ± 0.58
	Diazepam	10.91 ± 0.59	3.68 ± 0.15	5.55 ± 0.31	8.76 ± 0.32	13.23 ± 0.74	150.91 ± 2.85
	CF	11.81 ± 0.32	1.63 ± 0.14	1.88 ± 0.19	3.87 ± 0.33	4.49 ± 0.44	115.97 ± 1.55

Table 4.2 shows every single transport study ordered according to the fastness ranking of the NSAIDs substances in the time interval 0-40 minutes.

Single studies/Time interval: 0-40 min.

Date	Substance	Ratio PE_{all}/CF	Ratio PE_{cell}/CF	Ratio PE_{all}/DA	Ratio PE_{cell}/DA	Ratio $PE_{all}DA/CF$	Ratio $PE_{cell}DA/CF$
27/11/2008	Piroxicam	0.51 ± 0.11	0.43 ± 0.11	1.87 ± 0.02	2.69 ± 0.15	3.79 ± 0.82	6.66 ± 2.28
4/12/2008	Tenoxicam	0.86 ± 0.09	0.81 ± 0.09	2.57 ± 0.09	3.67 ± 0.29	3.00 ± 0.25	4.58 ± 0.44
20/11/2008	Ibuprofen	0.85 ± 0.09	0.76 ± 0.10	2.54 ± 0.13	3.84 ± 0.37	3.02 ± 0.31	5.14 ± 0.87
18/12/2008	Lornoxicam	1.34 ± 0.11	1.35 ± 0.13	3.36 ± 0.06	4.93 ± 0.39	2.52 ± 0.25	3.69 ± 0.66
11/12/2008	Meloxicam	1.09 ± 0.21	1.06 ± 0.24	3.13 ± 0.24	5.07 ± 0.88	2.92 ± 0.36	4.86 ± 0.49
13/11/2008	Diclofenac	1.41 ± 0.11	1.41 ± 0.13	4.61 ± 0.83	8.26 ± 1.01	3.25 ± 0.37	5.87 ± 0.56
24/2/2009	Celecoxib	5.44 ± 1.15	6.08 ± 1.38	12.22 ± 1.59	17.74 ± 2.18	2.28 ± 0.29	2.98 ± 0.47

Table 4.3 Ranking according to ratios diazepam and CF/substance in the time interval 0-40 minutes.

4.1.2 Time interval 40-240 minutes

Second part of interest was to analyse the time interval 40-240 minutes. As before, at first PS and PE data were compared. According to the Table 4.4 the ranking of all substances was the following:

diazepam → piroxicam → ibuprofen → tenoxicam → lornoxicam ⟶ meloxicam → diclofenac → carboxyfluorescein (CF)⟶ celecoxib

Here we can also say that the fastest substance was diazepam and the slowest one celecoxib. Nevertheless, at the first sight it was significant that CF has slowed down and now was the second slowest substance. Although that CF was passing the barrier faster than at the beginning (0-40 min.), it was slower in comparison to the other substances. Another change in the ranking was that the substances lornoxicam – meloxicam have switched the positions. Again, their numeral values were very similar and their standard deviations overlapped.

Analysing Table 4.5 with the ratio values differences can be observed compared to the time interval 0-40 min. The following ranking was obtained after normalisation to diazepam:

piroxicam → ibuprofen → tenoxicam → lornoxicam → celecoxib → meloxicam → diclofenac

This ranking also matched with the ranking of PE_{cell} values except the position of celecoxib. In relation to diazepam, celecoxib seems to be faster than meloxicam and diclofenac. However, it is necessary to mention that the PE_{cell} value of diazepam (19.93) in the single celecoxib study was significantly lower than PE_{cell} values of diazepam in the other single studies (between 24.86 and 39.95). This explains that the ratio was smaller and put the celecoxib further in the ranking. It is possible that celecoxib influenced diazepam because CF PE_{cell} values seemed to be very similar to other PE_{cell} values.

By observing the other ratio – substance/CF - another ranking was obtained:

piroxicam → ibuprofen → tenoxicam → diclofenac →meloxicam → lornoxicam → celecoxib

Here, celecoxib is the slowest substance again and the first three fastest substances were the same as before. The change is in the middle of the list, where diclofenac skipped over

meloxicam and lornoxicam. These two substances - meloxicam and lornoxicam - replaced each other's position.

In this time interval (40-240 min.) we noted many changes due to the different point of view. Only three substances – piroxicam, ibuprofen and tenoxicam – stayed in their positions on the top of the ranking.

Single studies/Time interval: 40-240 min.

Date	Substance	PS_{blank} [µl/min]	PS_{all} [µl/min]	PS_{cell} [µl/min]	PE_{all} [µm/min]	PE_{cell} [µm/min]	EoC [%]
27/11/2008	Piroxicam	8.53 ± 0.36	3.21 ± 0.07	5.15 ± 0.19	7.65 ± 0.18	12.27 ± 0.46	160.43 ± 2.25
	Diazepam	10.13 ± 0.43	5.57 ± 0.02	12.39 ± 0.11	13.26 ± 0.05	29.49 ± 0.27	222.33 ± 1.10
	CF	10.24 ± 0.30	1.60 ± 0.06	1.90 ± 0.09	3.81 ± 0.15	4.52 ± 0.20	118.54 ± 0.84
20/11/2008	Ibuprofen	5.54 ± 0.71	2.02 ± 0.02	3.19 ± 0.05	4.82 ± 0.05	7.60 ± 0.13	157.64 ± 0.99
	Diazepam	8.21 ± 0.29	4.60 ± 0.13	11.43 ± 0.68	10.94 ± 0.32	24.86 ± 1.63	227.11 ± 8.32
	CF	10.30 ± 1.45	1.54 ± 0.11	1.81 ± 0.15	3.67 ± 0.26	4.32 ± 0.36	117.61 ± 1.49
4/12/2008	Tenoxicam	7.12 ± 0.50	2.16 ± 0.02	3.11 ± 0.04	5.15 ± 0.05	7.40 ± 0.10	143.61 ± 0.56
	Diazepam	8.92 ± 0.72	4.97 ± 0.08	11.21 ± 0.41	11.83 ± 0.19	26.71 ± 0.98	225.73 ± 4.62
	CF	11.58 ± 0.67	1.58 ± 0.08	1.83 ± 0.11	3.77 ± 0.19	4.36 ± 0.26	115.82 ± 0.94
18/12/2008	Lornoxicam	6.54 ± 0.40	1.86 ± 0.05	2.59 ± 0.10	4.42 ± 0.12	6.18 ± 0.24	139.67 ± 1.56
	Diazepam	9.32 ± 0.35	5.05 ± 0.11	11.03 ± 0.51	12.03 ± 0.26	26.28 ± 1.21	218.43 ± 5.47
	CF	11.13 ± 0.44	1.76 ± 0.12	2.09 ± 0.17	4.19 ± 0.29	4.99 ± 0.40	118.81 ± 1.52
11/12/2008	Meloxicam	5.80 ± 0.26	1.77 ± 0.13	2.56 ± 0.27	4.23 ± 0.32	6.10 ± 0.65	144.21 ± 4.72
	Diazepam	7.49 ± 0.45	4.81 ± 0.14	13.40 ± 1.14	11.44 ± 0.34	32.00 ± 2.71	279.37 ± 15.19
	CF	9.70 ± 0.66	1.61 ± 0.004	1.92 ± 0.01	3.82 ± 0.01	4.58 ± 0.01	119.83 ± 0.06
13/11/2008	Diclofenac	4.08 ± 0.41	1.50 ± 0.08	2.38 ± 0.19	3.58 ± 0.18	5.67 ± 0.46	158.33 ± 4.70
	Diazepam	6.46 ± 1.01	4.66 ± 0.15	16.67 ± 1.88	11.09 ± 0.36	39.95 ± 4.48	359.71 ± 29.13
	CF	8.14 ± 0.30	1.36 ± 0.19	1.63 ± 0.28	3.23 ± 0.46	3.89 ± 0.67	120.10 ± 3.46
24/2/2009	Celecoxib	5.10 ± 0.06	1.39 ± 0.17	1.91 ± 0.32	3.31 ± 0.41	4.57 ± 0.76	137.68 ± 6.29
	Diazepam	10.20 ± 1.48	4.58 ± 0.34	8.32 ± 1.12	10.91 ± 0.80	19.93 ± 2.67	182.05 ± 10.98
	CF	11.45 ± 0.89	1.88 ± 0.08	2.25 ± 0.11	4.47 ± 0.18	5.35 ± 0.26	119.63 ± 0.97

Table 4.4 shows every single transport study ordered according to the fastness ranking of the NSAIDs substances in the time interval 40-240 minutes.

Single studies/Time interval: 40-240 min.

Date	Substance	Ratio PE_{all}/CF	Ratio PE_{cell}/CF	Ratio PE_{all}/DA	Ratio PE_{cell}/DA	Ratio $PE_{all}DA/CF$	Ratio $PE_{cell}DA/CF$
27/11/2008	Piroxicam	0.50 ± 0.01	0.37 ± 0.01	1.74 ± 0.03	2.41 ± 0.07	3.48 ± 0.12	6.53 ± 0.24
20/11/2008	Ibuprofen	0.76 ± 0.05	0.57 ± 0.04	2.27 ± 0.07	3.27 ± 0.22	2.99 ± 0.27	5.79 ± 0.76
4/12/2008	Tenoxicam	0.73 ± 0.04	0.59 ± 0.04	2.30 ± 0.06	3.61 ± 0.18	3.15 ± 0.16	6.14 ± 0.42
18/12/2008	Lornoxicam	0.95 ± 0.05	0.81 ± 0.05	2.72 ± 0.05	4.25 ± 0.14	2.88 ± 0.21	5.29 ± 0.51
24/2/2009	Celecoxib	1.36 ± 0.14	1.19 ± 0.17	3.31 ± 0.22	4.38 ± 0.30	2.44 ± 0.16	3.72 ± 0.46
11/12/2008	Meloxicam	0.91 ± 0.07	0.76 ± 0.09	2.72 ± 0.21	5.28 ± 0.63	2.99 ± 0.09	6.99 ± 0.60
13/11/2008	Diclofenac	0.91 ± 0.18	0.70 ± 0.18	3.11 ± 0.24	7.10 ± 1.22	3.47 ± 0.40	10.35 ± 1.05

Table 4.5 Ranking according to ratios diazepam and CF/substance in the time interval 40-240 minutes.

4.1.3 Acceleration factor

We can compare the PE_{cell} values from the two time periods and found out several correlations. Diazepam has speeded up about a factor 1.6 (from the first time interval until the second time interval). CF has speeded up about factor 1.2, which was the slowest factor and that made its drift down in the ranking. The other substances like piroxicam, ibuprofen and tenoxicam had more or less a similar acceleration factor, which was between 1.5 and 1.7. Lornoxicam speeded up almost about double (1.9) and diclofenac even more than double (2.3). The most significant acceleration was by celecoxib, which was about 6.1. This was maybe because the cell layers had to adopt their transporter systems, which was possibly induced by the presence of celecoxib. This was especially seen in the time intervals between 0-120 min., where the permeability of celecoxib steadily increased. Another possible reason was that celecoxib was toxic at 100 µM. In this regard, Germann (part of Neuhaus et al., 2008b) showed that 26 µM celecoxib did not change the amount of proteins or in particular ICAM-1 in ECV304 cells, but 100 µM celecoxib resulted in a reduction of proliferation (Szkokan, 2009). However, toxicity should also result in a loss of tightness, which was not observed considering the data of the paracellular marker CF. In the following graph (Figure 4.1) the acceleration of substances is shown according to different time interval and calculated as reciprocal values of ratio substance/diazepam.

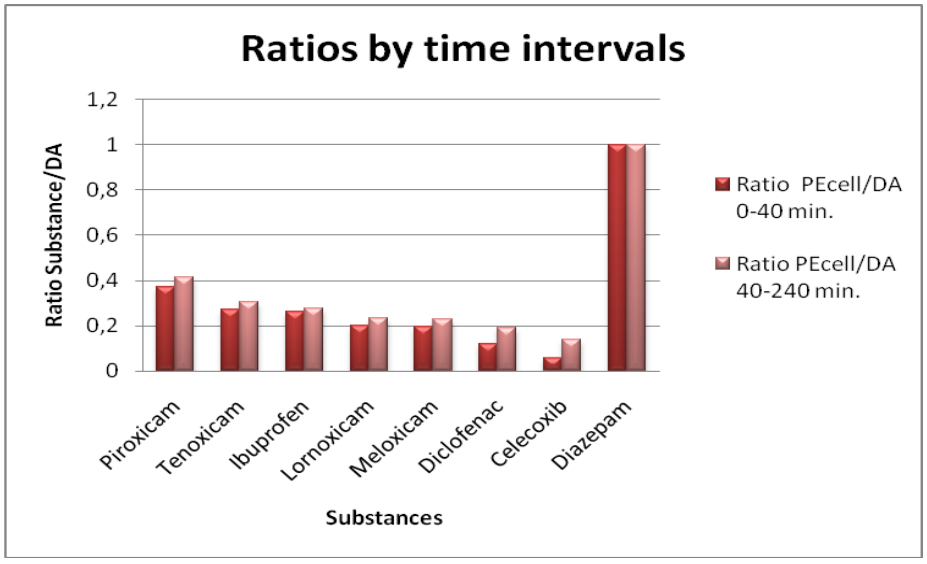


Figure 4.1 Ratios by time intervals
The graph shows ratios in two time intervals and their differences in the permeation speed through the barrier.

4.2 Group studies

Group studies were carried out to provide the same conditions for all substances at once. This offer a view on the possibility of substances to influence the transport properties of each other. To observe the behaviour of each substance better, group studies were carried out under different conditions. This may also show the potential interactions within substances. Two time intervals (0-40, 40-240 min.) were also distinguished.

4.2.1 Time interval 0-40 minutes

To be able to compare the group studies properly the data of different studies were grouped. The criterion for splitting them was to ensure the same conditions under which the experiments were carried out. Two major studies were picked up to be the referential studies. The first one was the study with 1 % DMSO including ibuprofen, diclofenac, meloxicam, tenoxicam, piroxicam and internal standards, because also 1 % DMSO was used in all single studies, so more data could be compared. The other referential study was also with 1 % DMSO, but without meloxicam and without any blockers. This study was the reference for studies with verapamil and probenecid. Finally, the study with 1 % DMSO, included all six substances (that means including celecoxib) was compared with these two referential studies and thereby presented a kind of transition between these two group studies. PE_{cell} values used for the comparisons were listed in the following Tables 4.6, 4.7 and 4.8.

- *Study with 1 % DMSO vs. 0.1 % DMSO*

Using DMSO (dimethylsulfoxid) was necessary for dissolving some substances (especially those, which were not obtained as a salt, e.g. piroxicam, tenoxicam and blockers etc.). It was sure that DMSO does not respond with the human settings so the best effort was to use as low DMSO amount as possible. Thus, 1 % DMSO was settled as the maximum, which should be used. Ranking of the referential study with 1 % DMSO was:

diazepam → piroxicam → ibuprofen → meloxicam → tenoxicam → diclofenac → carboxyfluorescein (CF)

To see the influence of this solvent a study with 0.1 % DMSO was carried out. The permeability coefficients PE_{cell} of the 0.1 % DMSO study were a little bit higher but the ranking of substances was the same. However, the differences were not significant.

diazepam → piroxicam → ibuprofen → meloxicam → tenoxicam → diclofenac → carboxyfluorescein (CF)

As we can see, diazepam was the fastest substance, followed by piroxicam and ibuprofen and the slowest substance was CF.

- *Study with 1 % DMSO vs. study with minus Fib medium*

Further, a study, whereas the main vehicle medium minus Fib medium was used, was compared with to referential study with 1 % DMSO. This should have shown the differences between the two used media in general. In this case, PE_{cell} values have differed a bit more. The ranking has also changed:

diazepam → piroxicam → tenoxicam → ibuprofen → meloxicam → carboxyfluorescein (CF) → diclofenac

Remarkably, the fastest substances were diazepam and piroxicam again. Tenoxicam over-jumped ibuprofen and meloxicam. Nevertheless, their PE_{cell} values were very close to each other (6.53 – 6.28 – 6.27). The end of the ranking has also changed in comparison to the study with 1 % DMSO, CF was faster than diclofenac. PE_{cell} of CF was quite similar to the value by the study with 1 % DMSO. On the other hand, diclofenac showed serious deceleration. Maybe this was also due to a change of a transporter system specific for diclofenac because of the lack of astrocytic factors.

- *Study with 1 % DMSO vs. study with medium without serum*

As we know, NSAIDs bind to plasma proteins from 98-99 %, which represents a very high amount. As we expected, this study showed many differences. The ranking has changed and the PE_{cell} values were significantly increased.

diazepam → ibuprofen → piroxicam → diclofenac → meloxicam → carboxyfluorescein (CF) → tenoxicam

According to the PE_{cell} values, all substances have speeded up at least about factor 1.3 – 1.6. Ibuprofen was almost double so fast (1.9) as in the 1 % DMSO study and

diclofenac even more than double and a half (2.6). Interestingly, especially substances with a carboxylic group (ibuprofen, diclofenac, CF) permeated significantly faster. This could be linked as a hint to the knowledge that ibuprofen (unknown) and CF (MRPs) should be recognised by active transporter systems. Although tenoxicam was here on the last position in the ranking, the PE_{cell} values with CF were very close.

- *Study with 1 % DMSO vs. study without CF*

It was known that CF could also bind to plasma proteins. Hence, study excluding CF was carried out. It showed some influence on the ranking:

diazepam → piroxicam → meloxicam → tenoxicam → ibuprofen → diclofenac

Although the first positions belonged to diazepam and piroxicam again, their PE_{cell} values were a bit lower. Surprisingly, meloxicam and tenoxicam overtook ibuprofen, but it was not because of increase of their PE_{cell} values because they stayed similar as by 1 % DMSO study. Conversely, values of ibuprofen and diclofenac have decreased rapidly. This may show the very high ability of these substances to bind to plasma proteins. Hence, of increased binding possibility, the free fraction decreased and so less amount of the substance was available for passing the barrier. Since carboxyfluorescein possesses also a carboxylic group as ibuprofen and diclofenac do, this maybe reflected the possible interactions between these substances.

- *Study with 1 % DMSO vs. study included celecoxib*

Celecoxib was involved in later studies, which provided new data to compare.

diazepam → piroxicam → ibuprofen → tenoxicam → meloxicam → carboxyfluorescein (CF) → diclofenac → celecoxib

Diazepam, piroxicam and ibuprofen were still on the top of the ranking although the PE_{cell} values of piroxicam and ibuprofen were lower compared to 1 % DMSO study. Tenoxicam switched with meloxicam, which value was a bit decreased. CF values were also almost the same as before, but diclofenac was lower and was placed behind CF. The slowest substance was celecoxib with PE_{cell} value 1.66 only.

- *Study incl. celecoxib vs. study without blockers and without meloxicam*

These two studies represented a kind of connection, which can be compared to observe the influence of one missing substance on the transport ability of the other substances. The ranking by the study without meloxicam and blockers was following:

diazepam → piroxicam → ibuprofen → tenoxicam → diclofenac → carboxyfluorescein (CF) → celecoxib

The substances generally permeated about 2-fold faster (celecoxib 2.0; piroxicam 1.9; diazepam 1.5 etc.) and diclofenac has overtaken CF. Nevertheless, the first four substances were in the same ranking as in the study including celecoxib. Thus, it seemed that meloxicam was able to influence the transport rates especially of celecoxib and piroxicam.

- *Study without blockers and without meloxicam vs. study with probenecid and without meloxicam*

Using transport mechanism's blocker has a huge significance for better understanding the transport systems and possible influences. Ranking influenced by probenecid was following:

diazepam → piroxicam → ibuprofen → diclofenac → carboxyfluorescein (CF) → tenoxicam → celecoxib

By using probenecid the PE_{cell} values were generally decreased than without a blocker. The first three substances stayed on their positions as before. The most significant deceleration showed tenoxicam (f = 3.3) and this placed it behind diclofenac and CF but still before celecoxib. In addition, celecoxib decelerated almost double (1.8). Thus, probenecid maybe influenced the transport of tenoxicam and celecoxib significantly.

- *Study without blockers and without meloxicam vs. study with verapamil and without meloxicam*

Verapamil blocked another types of transport systems than probenecid so differences in the ranking were expected.

diazepam → piroxicam → tenoxicam → ibuprofen → carboxyfluorescein (CF) → diclofenac → celecoxib

The PE_{cell} values were decreased for all substances. The relevant deceleration factor was found out by ibuprofen – 1.6; diclofenac – 1.8 and also by celecoxib – 2.7. The deceleration factors were even higher than in the study with probenecid. Since verapamil influences or acts as substrate of OCTs and other transporters (H^+/tert. amine antiporter; P-gp), this is maybe reflected in the changes of the permeability coefficients. However, verapamil is also known to decrease the tightness by modulating the intracellular Ca^{2+}-level. In contrast to this, no change in the permeability of CF was detected.

Group studies/Time interval: 0-40 min.

Date & Study	Substance	PS_{blank} [µl/min]	PS_{all} [µl/min]	PS_{cell} [µl/min]	PE_{all} [µm/min]	PE_{cell} [µm/min]	EoC [%]
3/2/2009 **1% DMSO**	Diazepam	10.13 ± 0.29	4.15 ± 0.50	7.04 ± 1.49	9.89 ± 1.19	16.95 ± 3.51	170.27 ± 14.56
	Piroxicam	10.74 ± 0.19	3.44 ± 0.32	5.07 ± 0.71	8.20 ± 0.77	12.12 ± 1.69	147.36 ± 6.59
	Ibuprofen	9.85 ± 0.33	2.75 ± 0.35	3.82 ± 0.68	6.55 ± 0.82	9.14 ± 1.63	138.98 ± 6.95
	Meloxicam	10.46 ± 0.23	2.38 ± 0.24	3.08 ± 0.40	5.67 ± 0.57	7.36 ± 0.96	129.55 ± 3.86
	Tenoxicam	10.32 ± 0.11	2.26 ± 0.16	2.89 ± 0.27	5.37 ± 0.39	6.88 ± 0.63	128.00 ± 2.57
	Diclofenac	9.40 ± 0.31	1.75 ± 0.31	2.16 ± 0.48	4.18 ± 0.74	5.17 ± 1.14	123.09 ± 5.10
	CF	11.45 ± 0.44	1.66 ± 0.06	1.94 ± 0.09	3.94 ± 0.15	4.61 ± 0.21	116.91 ± 0.77
10/2/2009 **0.1% DMSO**	Diazepam	9.58 ± 0.42	4.53 ± 0.01	8.60 ± 0.04	10.79 ± 0.03	20.48 ± 0.10	189.82 ± 0.44
	Piroxicam	10.47 ± 0.35	3.71 ± 0.02	5.73 ± 0.04	8.82 ± 0.04	13.65 ± 0.09	154.78 ± 0.38
	Ibuprofen	9.64 ± 0.43	2.78 ± 0.22	3.90 ± 0.43	6.61 ± 0.52	9.30 ± 1.01	140.51 ± 4.41
	Meloxicam	10.03 ± 0.39	2.47 ± 0.02	3.28 ± 0.04	5.89 ± 0.05	7.82 ± 0.09	132.77 ± 0. 39
	Tenoxicam	10.16 ± 0.32	2.42 ± 0.04	3.17 ± 0.09	5.76 ± 0.09	7.56 ± 0.15	131.25 ± 0.64
	Diclofenac	8.87 ± 0.67	1.78 ± 0.01	2.22 ± 0.01	4.23 ± 0.02	5.29 ± 0.02	125.04 ± 0.12
	CF	10.28 ± 0.35	1.76 ± 0.04	2.13 ± 0.06	4.20 ± 0.10	5.07 ± 0.14	120.70 ± 0.57
17/2/2009 **minus Fib medium**	Diazepam	9.95 ± 0.35	5.36 ± 0.17	11.63 ± 0.79	12.77 ± 0.41	27.74 ± 1.89	217.15 ± 7.98
	Piroxicam	9.55 ± 0.82	3.47 ± 0.18	5.46 ± 0.43	8.27 ± 0.42	13.02 ± 1.02	157.27 ± 4.51
	Tenoxicam	9.51 ± 0.31	2.13 ± 0.07	2.74 ± 0.12	5.07 ± 0.18	6.53 ± 0.29	128.84 ± 1.28
	Ibuprofen	8.71 ± 0.43	2.02 ± 0.01	2.64 ± 0.01	4.82 ± 0.02	6.28 ± 0.03	130.27 ± 0.16
	Meloxicam	9.52 ± 0.27	2.06 ± 0.09	2.63 ± 0.15	4.91 ± 0.21	6.27 ± 0.35	127.67 ± 1.53
	CF	10.01 ± 0.29	1.73± 0.25	2.09 ± 0.37	4.12 ± 0.58	4.99 ± 0.87	120.95 ± 3.65
	Diclofenac	8.30 ± 0.36	1.36 ± 0.11	1.63 ± 0.16	3.24 ± 0.27	3.87 ± 0.38	119.61 ± 1.93
3/3/2009 **without serum**	Diazepam	11.00 ± 0.67	5.10 ± 0.30	9.51 ± 1.00	12.14 ± 0.71	22.73 ± 2.39	186.81 ± 9.14
	Ibuprofen	11.01 ± 0.76	4.37 ± 0.15	7.24 ± 0.41	10.40 ± 0.36	17.25 ± 0.99	165.78 ± 3.76
	Piroxicam	10.83 ± 0.69	3.95 ± 0.20	6.23 ± 0.50	9.41 ± 0.48	14.85 ± 1.18	157.63 ± 4.58
	Diclofenac	10.95 ± 0.67	3.71 ± 0.23	5.62 ± 0.51	8.84 ± 0.54	13.40 ± 1.21	151.37 ± 4.64
	Meloxicam	10.77 ± 0.74	3.34 ± 0.19	4.84 ± 0.41	7.95 ± 0.46	11.54 ± 0.97	144.98 ± 3.79
	CF	11.34 ± 0.40	2.40 ± 0.06	3.05 ± 0.09	5.72 ± 0.13	7.26 ± 0.21	126.89 ± 0.78
	Tenoxicam	9.36 ± 0.46	2.25 ± 0.39	2.96 ± 0.70	5.35 ± 0.93	7.11 ± 1.66	131.90 ± 7.46

Table 4.6 shows every group transport study ordered according to the fastness of the NSAIDs substances within a study in the time interval 0-40 minutes.

Group studies/Time interval: 0-40 min.

Date & Study	Substance	PS_{blank} [µl/min]	PS_{all} [µl/min]	PS_{cell} [µl/min]	PE_{all} [µm/min]	PE_{cell} [µm/min]	EoC [%]
10/3/2009 **without CF**	Diazepam	10.53 ± 0.73	3.99 ± 0.11	6.43 ± 0.29	9.51 ± 0.26	15.32 ± 0.68	161.08 ± 2.72
	Piroxicam	11.03 ± 0.67	3.25 ± 0.05	4.62 ± 0.11	7.75 ± 0.13	10.99 ± 0.26	141.84 ± 0.97
	Meloxicam	10.85 ± 0.67	2.38 ± 0.10	3.06 ± 0.16	5.68 ± 0.23	7.28 ± 0.38	128.19 ± 1.47
	Tenoxicam	11.02 ± 0.33	2.27 ± 0.51	2.86 ± 0.80	5.40 ± 1.21	6.88 ± 1.90	126.21 ± 7.23
	Ibuprofen	10.16 ± 0.72	2.22 ± 0.13	2.83 ± 0.21	5.28 ± 0.30	6.75 ± 0.49	127.92 ± 2.04
	Diclofenac	9.79 ± 0.77	1.61 ± 0.14	1.93 ± 0.21	3.84 ± 0.34	4.60 ± 0.49	119.71 ± 2.11
	CF	-	-	-	-	-	-
17/3/2009 **with Celecoxib**	Diazepam	10.31 ± 0.52	4.34 ± 0.11	7.52 ± 0.31	10.35 ± 0.25	17.90 ± 0.74	172.96 ± 3.02
	Piroxicam	9.47 ± 1.40	2.71 ± 0.25	3.79 ± 0.49	6.44 ± 0.58	9.05 ± 1.17	140.13 ± 5.19
	Ibuprofen	8.50 ± 0.57	2.28 ± 0.20	3.12 ± 0.36	5.44 ± 0.47	7.45 ± 0.86	136.81 ± 4.25
	Tenoxicam	10.54 ± 0.56	2.37 ± 0.09	3.06 ± 0.15	5.65 ± 0.22	7.30 ± 0.36	129.07 ± 1.43
	Meloxicam	10.46 ± 0.71	2.28 ± 0.15	2.91 ± 0.24	5.42 ± 0.36	6.93 ± 0.58	127.82 ± 2.33
	CF	11.10 ± 0.63	1.72 ± 0.23	2.04 ± 0.31	4.10 ± 0.54	4.87 ± 0.75	118.44 ± 2.82
	Diclofenac	8.89 ± 0.22	1.60 ± 0.14	1.95 ± 0.20	3.81 ± 0.32	4.65 ± 0.48	121.97 ± 2.26
	Celecoxib	7.79 ± 0.63	0.64 ± 0.04	0.70 ± 0.04	1.53 ± 0.09	1.66 ± 0.11	108.97 ± 0.58

Table 4.7 Ranking according to the fastness of the NSAIDs substances within a study in the time interval 0-40 minutes.

Group studies/Time interval: 0-40 min.

Date & Study	Substance	PS_{blank} [µl/min]	PS_{all} [µl/min]	PS_{cell} [µl/min]	PE_{all} [µm/min]	PE_{cell} [µm/min]	EoC [%]
21/4/2009	Diazepam	9.36 ± 0.25	5.13 ± 0.16	11.38 ± 0.79	12.23 ± 0.38	27.14 ± 1.88	221.85 ± 8.45
without	Piroxicam	9.93 ± 0.26	4.12 ± 0.07	7.03 ± 0.21	9.80 ± 0.17	16.75 ± 0.50	170.85 ± 2.13
bloskers	Ibuprofen	8.56 ± 0.19	2.78 ± 0.11	4.12 ± 0.23	6.63 ± 0.25	9.82 ± 0.56	148.19 ± 2.74
without	Tenoxicam	9.28 ± 0.31	2.64 ± 0.03	3.68 ± 0.07	6.28 ± 0.08	8.77 ± 0.16	139.68 ± 0.72
Melox	Diclofenac	8.13 ± 0.10	2.03 ± 0.04	2.71 ± 0.07	4.84 ± 0.10	6.45 ± 0.17	133.34 ± 0.88
	CF	9.44 ± 0.11	1.78 ± 0.10	2.20 ± 0.15	4.24 ± 0.23	5.23 ± 0.23	123.27 ± 1.56
	Celecoxib	6.73 ± 0.15	1.13 ± 0.13	1.36 ± 0.18	2.69 ± 0.30	3.24 ± 0.43	120.21 ± 2.71
	Meloxicam	-	-	-	-	-	-
24/3/2009	Diazepam	9.06 ± 0.22	4.40 ± 0.10	8.57 ± 0.39	10.49 ± 0.25	20.42 ± 0.93	194.68 ± 4.33
with	Piroxicam	9.28 ± 0.22	3.26 ± 0.16	5.02 ± 0.38	7.76 ± 0.38	11.98 ± 0.90	154.22 ± 4.06
Probenecid	Ibuprofen	8.17 ± 0.15	2.09 ± 0.33	2.81 ± 0.59	4.98 ± 0.79	6.74 ± 1.39	134.65 ± 7.17
without	Diclofenac	7.96 ± 0.16	1.74 ± 0.20	2.22 ± 0.33	4.13 ± 0.48	5.30 ± 0.79	127.97 ± 4.18
Melox	CF	9.77± 0.21	1.52 ± 0.04	1.80 ± 0.06	3.61 ± 0.11	4.28 ± 0.15	118.37 ± 0.64
	Tenoxicam	8.41 ± 0.91	0.97 ± 0.58	1.09 ± 0.71	2.30 ± 1.38	2.68 ± 1.70	113.40 ± 8.49
	Celecoxib	6.80 ± 0.04	0.69 ± 0.06	0.77 ± 0.08	1.65 ± 0.15	1.84 ± 0.18	111.37 ± 1.14
	Meloxicam	-	-	-	-	-	-
31/3/2009	Diazepam	8.24 ± 0.07	4.02 ± 0.24	7.84 ± 0.92	9.56 ± 0.56	18.74 ± 2.19	195.58 ± 11.15
with	Piroxicam	7.96 ± 0.69	2.67 ± 0.17	4.01 ± 0.40	6.35 ± 0.42	9.56 ± 0.95	150.43 ± 5.01
Verapamil	Tenoxicam	8.08 ± 0.49	1.99 ± 0.04	2.64± 0.07	4.74 ± 0.10	6.29 ± 0.17	132.67 ± 0.90
without	Ibuprofen	5.49 ± 0.13	1.72 ± 0.23	2.49 ± 0.48	4.09 ± 0.54	5.98 ± 1.15	145.77 ± 8.77
Melox	CF	9.17 ± 0.06	1.58 ± 0.18	1.90 ± 0.26	3.75 ± 0.42	4.54 ± 0.62	120.79 ± 2.84
	Diclofenac	6.15 ± 0.76	1.22 ± 0.05	1.52 ± 0.07	2.90 ± 0.11	3.61 ± 0.17	124.67 ± 1.16
	Celecoxib	5.33 ± 0.48	0.47 ± 0.05	0.51 ± 0.06	1.11 ± 0.12	1.22 ± 0.15	109.64 ± 1.16
	Meloxicam	-	-	-	-	-	-

Table 4.8 shows every group transport study ordered according to the fastness of the NSAIDs substances within a study in the time interval 0-40 minutes.

When we confronted the obtained PE_{cell} data and their ranking by every group study with the ratio data in relation to diazepam and CF, we found many correlations (Tables 4.9 and 4.10).

By studies:

- with 1 % DMSO
- with 0.1 % DMSO
- with medium without serum
- included celecoxib
- without blockers and without meloxicam
- with verapamil and without meloxicam

the ratios DA/substance and substance/CF showed exactly the same ranking as the PE_{cell} values.

Small differences were found by the ***study with minus Fib medium***. The differences between the PE_{cell} and the ratio rankings may result from the direct relation of the single values (from n = 3) to calculate the ratios. The ranking according to ratio DA/substances as well as according to CF was:

piroxicam → tenoxicam → ibuprofen = meloxicam → diclofenac

Here, it is necessary to point out that ibuprofen and meloxicam share the same position and their values differ only in the standard deviations. Compared to the PE_{cell} values, it was observed that also there were the data very close to each other (ibuprofen = 6.28; meloxicam = 6.27).

Next differences were observed in the ***study without CF***. Ibuprofen overtook tenoxicam compared to the PE_{cell} ranking. However, with inclination to standard deviation we can say that the differences were not big and they overlapped.

piroxicam → meloxicam → ibuprofen → tenoxicam → diclofenac

The last study, which differed, was the ***study with probenecid*** and without meloxicam. Tenoxicam switched with celecoxib and became so the slowest substance. The ranking was following:

piroxicam → ibuprofen → diclofenac → celecoxib →tenoxicam

Group studies/Time interval: 0-40 min.

Date & Study	Substance	Ratio PE_{all}/CF	Ratio PE_{cell}/CF	Ratio PE_{all}/DA	Ratio PE_{cell}/DA	Ratio $PE_{all}DA/CF$	Ratio $PE_{cell}DA/CF$
3/2/2009 **1% DMSO**	Piroxicam	0.50 ± 0.04	0.41 ± 0.05	1.20 ± 0.03	1.39 ± 0.10	2.51 ± 0.37	3.69 ± 0.86
	Ibuprofen	0.61 ± 0.09	0.52 ± 0.10	1.51 ± 0.04	1.85 ± 0.09		
	Meloxicam	0.73 ± 0.06	0.67 ± 0.06	1.74 ± 0.04	2.29 ± 0.17		
	Tenoxicam	0.74 ± 0.08	0.68 ± 0.09	1.84 ± 0.09	2.45 ± 0.29		
	Diclofenac	0.96 ± 0.17	0.92 ± 0.20	2.38 ± 0.17	3.29 ± 0.22		
10/2/2009 **0.1% DMSO**	Piroxicam	0.48 ± 0.01	0.37 ± 0.01	1.22 ± 0.01	1.50 ± 0.02	2.57 ± 0.05	4.04 ± 0.10
	Ibuprofen	0.64 ± 0.07	0.55 ± 0.08	1.50 ± 0.19	2.22 ± 0.27		
	Meloxicam	0.71 ± 0.02	0.65 ± 0.02	1.83 ± 0.01	2.62 ± 0.02		
	Tenoxicam	0.73 ± 0.03	0.67 ± 0.03	1.87 ± 0.03	2.71 ± 0.07		
	Diclofenac	0.99 ± 0.02	0.96 ± 0.03	2.55 ± 0.02	3.87 ± 0.04		
17/2/2009 **minus Fib medium**	Piroxicam	0.50 ± 0.07	0.38 ± 0.07	1.54 ± 0.03	2.13 ± 0.03	3.14 ± 0.42	5.66 ± 0.96
	Tenoxicam	0.81 ± 0.11	0.76 ± 0.12	2.52 ± 0.02	4.25 ± 0.11		
	Meloxicam	0.84 ± 0.13	0.80 ± 0.15	2.60 ± 0.04	4.42 ± 0.10		
	Ibuprofen	0.85 ± 0.12	0.80 ± 0.14	2.65 ± 0.07	4.42 ± 0.28		
	Diclofenac	1.28 ± 0.21	1.30 ± 0.26	3.96 ± 0.20	7.18 ± 0.25		
3/3/2009 **without serum**	Ibuprofen	0.55 ± 0.03	0.42 ± 0.03	1.17 ± 0.07	1.32 ± 0.14	2.12 ± 0.16	3.14 ± 0.40
	Piroxicam	0.61 ± 0.04	0.49 ± 0.05	1.29 ± 0.03	1.53 ± 0.07		
	Diclofenac	0.65 ± 0.05	0.55 ± 0.07	1.37 ± 0.01	1.69 ± 0.03		
	Meloxicam	0.72 ± 0.06	0.63 ± 0.07	1.53 ± 0.05	1.97 ± 0.11		
	Tenoxicam	1.09 ± 0.20	1.06 ± 0.26	2.30 ± 0.28	3.27 ± 0.49		
10/3/2009 **without CF**	Piroxicam	-	-	1.23 ± 0.02	1.39 ± 0.04	-	-
	Meloxicam	-	-	1.68 ± 0.02	2.10 ± 0.02	-	-
	Ibuprofen	-	-	1.80 ± 0.05	2.27 ± 0.07	-	-
	Tenoxicam	-	-	1.82 ± 0.42	2.35 ± 0.67	-	-
	Diclofenac	-	-	2.49 ± 0.15	3.35 ± 0.21	-	-

Table 4.9 shows every group transport study ordered according to ratio values DA/substance in the time interval 0-40 minutes.

Group studies/Time interval: 0-40 min.

Date & Study	Substance	Ratio PE_{all}/CF	Ratio PE_{cell}/CF	Ratio PE_{all}/DA	Ratio PE_{cell}/DA	Ratio $PE_{all}DA/CF$	Ratio $PE_{cell}DA/CF$
17/3/2009 **with Celecoxib**	Piroxicam	0.64 ± 0.07	0.54 ± 0.08	1.61 ± 0.13	2.00 ± 0.23	2.55 ± 0.30	3.72 ± 0.46
	Ibuprofen	0.75 ± 0.04	0.65 ± 0.03	1.91 ± 0.12	2.42 ± 0.19		
	Tenoxicam	0.72 ± 0.07	0.67 ± 0.07	1.83 ± 0.03	2.45 ± 0.03		
	Meloxicam	0.76 ± 0.05	0.70 ± 0.05	1.91 ± 0.08	2.59 ± 0.12		
	Diclofenac	1.07 ± 0.05	1.04 ± 0.06	2.73 ± 0.18	3.87 ± 0.26		
	Celecoxib	2.68 ± 0.23	2.92 ± 0.31	6.79 ± 0.28	10.77 ± 0.42		
21/4/2009 **without blockers Melox/**	Piroxicam	0.43 ± 0.02	0.31 ± 0.02	1.25 ± 0.02	1.62 ± 0.07	2.88 ± 0.08	5.19 ± 0.13
	Ibuprofen	0.64 ± 0.04	0.53 ± 0.04	1.85 ± 0.06	2.76 ± 0.16		
	Tenoxicam	0.68 ± 0.03	0.60 ± 0.04	1.95 ± 0.05	3.09 ± 0.18		
	Diclofenac	0.88 ± 0.05	0.81 ± 0.05	2.53 ± 0.06	4.21 ± 0.23		
	Celecoxib	1.59 ± 0.09	1.63 ± 0.11	4.58 ± 0.38	8.44 ± 0.59		
	Meloxicam	-	-	-	-		
24/3/2009 **with Probenecid without Melox**	Piroxicam	0.47 ± 0.01	0.36 ± 0.02	1.35 ± 0.04	1.71 ± 0.05	2.90 ± 0.04	4.78 ± 0.11
	Ibuprofen	0.74 ± 0.11	0.65 ± 0.13	2.14 ± 0.32	3.11 ± 0.57		
	Diclofenac	0.88 ± 0.11	0.82 ± 0.12	2.56 ± 0.27	3.90 ± 0.53		
	Celecoxib	2.20 ± 0.19	2.34 ± 0.23	6.37 ± 0.47	11.14 ± 0.88		
	Tenoxicam	2.50 ± 2.33	2.76 ± 2.77	7.21 ± 6.62	13.09 ± 13.04		
	Meloxicam	-	-	-	-		
31/3/2009 **with Verapamil without Melox**	Piroxicam	0.59 ± 0.03	0.47 ± 0.02	1.51 ± 0.16	1.98 ± 0.37	2.57 ± 0.36	4.19 ± 0.88
	Tenoxicam	0.79 ± 0.07	0.72 ± 0.08	2.02 ± 0.12	2.98 ± 0.34		
	Ibuprofen	0.93 ± 0.13	0.77 ± 0.15	2.38 ± 0.46	3.26 ± 1.01		
	Diclofenac	1.29 ± 0.12	1.26 ± 0.15	3.31 ± 0.32	5.22 ± 0.86		
	Celecoxib	3.37 ± 0.03	3.71 ± 0.07	8.67 ± 1.24	15.54 ± 3.17		
	Meloxicam	-	-	-	-		

Table 4.10 shows every group transport study ordered according to ratio values DA/substance in the time interval 0-40 minutes.

4.2.2. Time interval 40-240 minutes

The comparison followed in the same way as by the time interval 0-40 minutes. The PS and PE values are demonstrated in the Tables 4.11, 4.12 and 4.13.

The ranking by the first referential study with 1 % DMSO was:

diazepam → piroxicam → ibuprofen → meloxicam → tenoxicam → diclofenac → carboxyfluorescein (CF)

- *Study with 1 % DMSO vs. 0.1 % DMSO*

Here the ranking has not changed. The only observed point was the little increased PE_{cell} values of ibuprofen, diclofenac and CF. All other values were very similar.

- *Study with 1 % DMSO vs. study with minus Fib medium*

The first positions in the ranking were occupied again by diazepam, piroxicam and ibuprofen. PE_{cell} value of ibuprofen has decreased (from 14.24 to 8.59). Meloxicam changed position with tenoxicam, but their values were very similar. Generally, all values decreased except diazepam and CF whose PE_{cell} values increased. This maybe reflects the influence of the presence of astrocytic factors, which can stabilise the BBB properties, especially the activity of some transporter systems.

diazepam → piroxicam → ibuprofen → tenoxicam → meloxicam → diclofenac → carboxyfluorescein (CF)

- *Study with 1 % DMSO vs. study with medium without serum*

As expected, the ranking in this study has changed dramatically:

diazepam → ibuprofen → diclofenac → piroxicam → meloxicam → tenoxicam → carboxyfluorescein (CF)

Ibuprofen and diclofenac were faster about a factor 2.0 and 2.7 and thus overtook piroxicam in the ranking. Generally, all substances had higher PE_{cell} values. Another

significant acceleration factor was to observe for CF (1.9). Here, also the substances possessing a carboxylic group were influenced mostly.

- *Study with 1 % DMSO vs. study without CF*

diazepam → piroxicam → tenoxicam → meloxicam → ibuprofen → diclofenac

PE_{cell} values of tenoxicam and meloxicam were increased so they replaced ibuprofen, which value has decreased. Nevertheless, values of ibuprofen and meloxicam were very close (Ibu = 12.05; Melox = 12.15). Diazepam and piroxicam stayed on the top again.

- *Study with 1 % DMSO vs. study included celecoxib*

The ranking of substances corresponded to the study with 1 % DMSO, therewith the additional celecoxib was on the place before CF. PE_{cell} values of piroxicam, ibuprofen and diclofenac were a bit decreased.

diazepam → piroxicam → ibuprofen → meloxicam → tenoxicam → diclofenac → celecoxib → carboxyfluorescein (CF)

- *Study incl. celecoxib vs. study without blockers and without meloxicam*

The beginning of the ranking is the same again whereas the values of PE_{cell} were increased (Diaz about factor 1.8; Pirox f = 2.1). Celecoxib and tenoxicam share the same position in the ranking, where celecoxib had an acceleration factor about 2.1.

diazepam → piroxicam → ibuprofen → celecoxib = tenoxicam → diclofenac → carboxyfluorescein (CF)

- *Study without blockers and without meloxicam vs. study with probenecid and without meloxicam*

Blocking transport systems led to general decrease of PE_{cell} values. The ranking started with the same fastest substances – diazepam, piroxicam and ibuprofen,

diclofenac and celecoxib have changed their places. The most significant deceleration was observed for tenoxicam, which slowed down about a factor 3.7 and this placed tenoxicam even behind CF. This may point to a fact, that probably tenoxicam has a higher affinity to some transport systems than other substances, which were inhibited by probenecid.

diazepam → piroxicam → ibuprofen → diclofenac → celecoxib → carboxyfluorescein (CF) → tenoxicam

- *Study without blockers and without meloxicam vs. study with verapamil and without meloxicam*

In addition, here was a significant decrease to observe. Ibuprofen was slower about a factor 1.7, but it still stayed on the third position. Further, permeation of celecoxib significantly decreased by a factor of 2.3 and that placed it to the second slowest place of the ranking. Nevertheless, except celecoxib the ranking stayed almost the same as by the study without blockers.

diazepam → piroxicam → ibuprofen → tenoxicam → diclofenac → celecoxib → carboxyfluorescein (CF)

Group studies/Time interval: 40-240 min.

Date & Study	Substance	PS_{blank} [µl/min]	PS_{all} [µl/min]	PS_{cell} [µl/min]	PE_{all} [µm/min]	PE_{cell} [µm/min]	EoC [%]
3/2/2009 **1% DMSO**	Diazepam	9.14 ± 0.53	4.78 ± 0.21	10.02 ± 0.59	11.38 ± 0.50	23.93 ± 2.16	209.95 ± 9.94
	Piroxicam	10.18 ± 0.61	4.22 ± 0.14	7.20 ± 0.42	10.04 ± 0.34	17.15 ± 1.00	170.72 ± 4.12
	Ibuprofen	8.27 ± 0.38	3.47 ± 0.08	5.98 ± 0.24	8.26 ± 0.19	14.24 ± 0.57	172.35 ± 2.92
	Meloxicam	9.54 ± 0.50	3.06 ± 0.07	4.50 ± 0.16	7.28 ± 0.18	10.73 ± 0.39	147.24 ± 1.70
	Tenoxicam	8.53 ± 0.52	2.79 ± 0.02	4.14 ± 0.04	6.64 ± 0.04	9.86 ± 0.09	148.53 ± 0.43
	Diclofenac	7.38 ± 0.30	2.50 ± 0.09	3.78 ± 0.21	5.96 ± 0.22	9.02 ± 0.50	151.31 ± 2.84
	CF	11.52 ± 1.33	1.73 ± 0.08	2.03 ± 0.10	4.11 ± 0.18	4.84 ± 0.25	117.64 ± 0.90
10/2/2009 **0.1% DMSO**	Diazepam	8.66 ± 0.24	4.62 ± 0.09	9.91 ± 0.43	11.00 ± 0.22	23.60 ± 1.02	214.48 ± 4.94
	Piroxicam	9.85 ± 0.39	4.27 ± 0.08	7.53 ± 0.25	10.16 ± 0.19	17.94 ± 0.59	176.45 ± 2. 52
	Ibuprofen	8.28 ± 0.29	3.21 ± 0.09	5.24 ± 0.25	7.64 ± 0.22	12.49 ± 0.60	163.33 ± 3.05
	Meloxicam	9.04 ± 0.56	3.01 ± 0.03	4.50 ± 0.06	7.16 ± 0.07	10.72 ± 0.15	149.82 ± 0.71
	Tenoxicam	9.07 ± 0.44	2.89 ± 0.03	4.25 ± 0.06	6.89 ± 0.06	10.12 ± 0.14	146.86 ± 0.64
	Diclofenac	6.58 ± 0.21	2.17 ± 0.07	3.24 ± 0.15	5.17 ± 0.16	7.73 ± 0.35	149.30 ± 2.22
	CF	9.76 ± 0.53	1.92 ± 0.10	2.39 ± 0.16	4.57 ± 0.25	5.69 ± 0.38	124.48 ± 1.63
17/2/2009 **minus Fib medium**	Diazepam	10.89 ± 0.78	6.50 ± 0.23	16.10 ± 1.37	15.47 ± 0.55	38.44 ± 3.27	248.27 ± 12.59
	Piroxicam	9.34 ± 1.52	4.22 ± 0.36	7.71 ± 1.23	10.06 ± 0.85	18.50 ± 0.93	183.23 ± 13.20
	Ibuprofen	6.64 ± 0.40	2.34 ± 0.08	3.60 ± 0.19	5.56 ± 0.19	8.59 ± 0.45	154.34 ± 2.84
	Tenoxicam	8.48 ± 0.51	2.45 ± 0.21	3.45 ± 0.41	5.84 ± 0.49	8.23 ± 0.99	140.78 ± 4.89
	Meloxicam	8.60 ± 0.46	2.46 ± 0.21	3.44 ± 0.43	5.85 ± 0.51	8.22 ± 1.02	140.13 ± 4.98
	Diclofenac	6.22 ± 0.35	1.77 ± 0.19	2.48 ± 0.38	4.22 ± 0.45	5.93 ± 0.91	139.99 ± 6.14
	CF	9.42 ± 0.41	1.89 ± 0.17	2.37 ± 0.27	4.50 ± 0.41	5.64 ± 0.63	125.14 ± 2.82
3/3/2009 **without serum**	Diazepam	8.92 ± 0.71	5.64 ± 0.26	15.32 ± 1.98	13.42 ± 0.62	36.72 ± 4.71	272.99 ± 22.18
	Ibuprofen	9.04 ± 0.98	5.11 ± 0.19	11.75 ± 1.02	12.16 ± 0.45	28.04 ± 2.44	230.30 ± 11.31
	Diclofenac	8.83 ± 0.84	4.73 ± 0.16	10.18 ± 0.74	11.26 ± 0.38	24.29 ± 1.77	215.57 ± 8.44
	Piroxicam	8.91 ± 0.69	4.60 ± 0.20	9.51 ± 0.85	10.95 ± 0.47	22.70 ± 2.03	206.95 ± 9.58
	Meloxicam	8.84 ± 0.75	4.07 ± 0.09	7.54 ± 0.32	9.69 ± 0.23	17.96 ± 0.77	185.31 ± 3.65
	Tenoxicam	5.93 ± 0.16	2.64 ± 0.38	4.76 ± 1.24	6.28 ± 0.90	11.55 ± 2.94	181.85 ± 20.86
	CF	10.24 ± 0.41	2.84 ± 0.09	3.94 ± 0.18	6.77 ± 0.22	9.38 ± 0.42	138.48 ± 1.73

Table 4.11 shows every group transport study ordered according to the fastness of the NSAIDs substances within a study in the time interval 40-240 minutes.

Group studies/Time interval: 40-240 min.

Date & Study	Substance	PS_{blank} [µl/min]	PS_{all} [µl/min]	PS_{cell} [µl/min]	PE_{all} [µm/min]	PE_{cell} [µm/min]	EoC [%]
10/3/2009 **without CF**	Diazepam	9.10 ± 0.70	5.00 ± 0.20	11.08 ± 0.98	11.90 ± 0.48	26.46 ± 2.33	222.10 ± 10.74
	Piroxicam	9.84 ± 0.78	4.30 ± 0.21	7.63 ± 0.63	10.23 ± 0.49	18.20 ± 1.51	177.67 ± 6.44
	Tenoxicam	8.99 ± 1.01	3.38 ± 0.38	5.40 ± 0.95	8.04 ± 0.91	12.97 ± 2.26	160.60 ± 10.56
	Meloxicam	9.62 ± 0.67	3.33 ± 0.11	5.10 ± 0.26	7.93 ± 0.27	12.15 ± 0.63	153.03 ± 2.75
	Ibuprofen	7.93 ± 0.74	3.08 ± 0.22	5.04 ± 0.60	7.34 ± 0.54	12.05 ± 1.42	163.86 ± 7.51
	Diclofenac	7.31 ± 0.53	2.60 ± 0.17	4.04 ± 0.41	6.20 ± 0.41	9.65 ± 0.97	155.49 ± 5.57
	CF	-	-	-	-	-	-
17/3/2009 **with Celecoxib**	Diazepam	9.45 ± 0.24	4.73 ± 0.33	9.49 ± 1.31	11.27 ± 0.79	22.74 ± 3.12	201.08 ± 13.87
	Piroxicam	6.60 ± 1.10	2.94 ± 0.34	5.31 ± 1.13	7.01 ± 0.80	12.81 ± 2.69	181.51 ± 17.10
	Ibuprofen	5.75 ± 0.05	2.53 ± 0.14	4.53 ± 0.45	6.03 ± 0.33	10.81 ± 1.07	178.97 ± 7.80
	Meloxicam	9.14 ± 0.27	2.89 ± 0.12	4.22 ± 0.26	6.87 ± 0.29	10.05 ± 0.63	146.18 ± 2.89
	Tenoxicam	9.70 ± 0.07	2.92 ± 0.09	4.19 ± 0.19	6.96 ± 0.22	9.97 ± 0.46	143.18 ± 1.99
	Diclofenac	6.46 ± 0.55	2.15 ± 0.11	3.23 ± 0.25	5.13 ± 0.26	7.70 ± 0.58	150.05 ± 3.79
	Celecoxib	5.44 ± 0.24	1.83 ± 0.10	2.76 ± 0.24	4.36 ± 0.25	6.58 ± 0.57	150.76 ± 4.38
	CF	11.15 ± 0.45	1.92 ± 0.14	2.31 ± 0.21	4.56 ± 0.34	5.51 ± 0.49	120.77 ± 1.84

Table 4.12 Ranking according to the fastness of the NSAIDs substances within a study in the time interval 40-240 minutes.

Group studies/Time interval: 40-240 min.

Date & Study	Substance	PS_{blank} [µl/min]	PS_{all} [µl/min]	PS_{cell} [µl/min]	PE_{all} [µm/min]	PE_{cell} [µm/min]	EoC [%]
21/4/2009	Diazepam	8.98 ± 0.78	5.87 ± 0.25	16.98 ± 2.16	13.99 ± 0.59	40.69 ± 5.13	290.25 ± 23.99
without	Piroxicam	10.08 ± 0.91	5.38 ± 0.16	11.54 ± 0.73	12.81 ± 0.37	27.52 ± 1.74	214.67 ± 7.24
blockers	Ibuprofen	6.87 ± 0.58	3.48 ± 0.12	7.06 ± 0.50	8.29 ± 0.29	16.85 ± 1.20	202.99 ± 7.32
without	Celecoxib	4.93 ± 0.48	2.68 ± 0.04	5.86 ± 0.17	6.38 ± 0.08	13.96 ± 0.41	218.89 ± 3.45
Melox	Tenoxicam	8.08 ± 0.66	3.40 ± 0.11	5.86 ± 0.36	8.09 ± 0.27	13.96 ± 0.80	172.56 ± 4.16
	Diclofenac	6.36 ± 0.63	2.89 ± 0.11	5.29 ± 0.37	6.88 ± 0.26	12.62 ± 0.89	183.43 ± 5.87
	CF	9.18 ± 0.81	1.87 ± 0.05	2.35 ± 0.08	4.45 ± 0.11	5.59 ± 0.18	125.55 ± 0.82
	Meloxicam	-	-	-	-	-	-
24/3/2009	Diazepam	8.08 ± 0.35	5.01 ± 0.01	13.21 ± 0.08	11.94 ± 0.03	31.46 ± 0.19	263.54 ± 1.01
with	Piroxicam	8.24 ± 0.35	3.90 ± 0.16	7.41 ± 0.58	9.29 ± 0.39	17.68 ± 1.39	190.14 ± 7.06
Probenecid	Ibuprofen	5.88 ± 0.23	2.68 ± 0.37	4.93 ± 1.19	6.39 ± 0.88	11.95 ± 2.83	185.33 ± 20.22
without	Diclofenac	5.90 ± 0.26	2.52 ± 0.13	4.41 ± 0.38	6.01 ± 0.31	10.53 ± 0.92	174.95 ± 6.52
Melox	Celecoxib	4.62 ± 0.25	2.11 ± 0.08	3.91 ± 0.25	5.04 ± 0.18	9.31 ± 0.60	184.67 ± 5.48
	CF	8.69 ± 0.61	1.72 ± 0.04	2.15 ± 0.06	4.10 ± 0.09	5.12 ± 0.14	124.73 ± 0.69
	Tenoxicam	5.27 ± 1.32	1.22 ± 0.20	1.58 ± 0.35	2.89 ± 0.48	3.79 ± 0.84	130.23 ± 6.73
	Meloxicam	-	-	-	-	-	-
31/3/2009	Diazepam	8.12 ± 0.40	4.58 ± 0.20	10.54 ± 1.04	10.91 ± 0.47	25.15 ± 2.47	230.16 ± 12.77
with	Piroxicam	6.76 ± 0.90	3.36 ± 0.10	6.68 ± 0.39	8.00 ± 0.23	15.93 ± 0.93	199.02 ± 5.78
Verapamil	Ibuprofen	4.09 ± 0.32	2.03 ± 0.23	4.04 ± 1.00	4.84 ± 0.56	9.80 ± 2.37	200.59 ± 24.30
without	Tenoxicam	7.20 ± 0.81	2.51 ± 0.02	3.86 ± 0.04	5.98 ± 0.04	9.18 ± 0.09	153.57 ± 0.53
Melox	Diclofenac	4.59 ± 0.29	1.83 ± 0.03	3.04 ± 0.07	4.35 ± 0.06	7.24 ± 0.17	166.22 ± 1.51
	Celecoxib	3.87 ± 0.26	1.56 ± 0.03	2.60 ± 0.08	3.71 ± 0.07	6.20 ± 0.19	167.36 ± 2.12
	CF	9.66 ± 0.33	1.70 ± 0.04	2.06 ± 0.06	4.05 ± 0.10	4.92 ± 0.15	121.37 ± 0.66
	Meloxicam	-	-	-	-	-	-

Table 4.13 shows every group transport study ordered according to the fastness of the NSAIDs substances within a study in the time interval 40-240 minutes.

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The overview of rankings of the ratios of each study reported total correspondences (Tables 4.15 and 4.16).

Some findings, which have to be mentioned, were found out in three studies. In two of them, meloxicam and tenoxicam had very close or even total similar values within each study, where they differed just in the range of the standard deviations. This was observed in the study, where ***minus Fib medium*** was used and in the group study, which ***included celecoxib***.

In the other case, there were correspondences between tenoxicam and celecoxib in the ***study without blockers*** and without meloxicam. The PE_{cell} values were totally the same.

Also here should not be forgotten the possible influence of TEER on the permeation abilities of the substances. The TEER values are shown in the Table 4.14 thereby the results of the studies can be correlated with the TEER values immediately before the studies. Considering the TEER values, the differences in the permeability coefficients of the charged substances containing a carboxylic acid (ibuprofen, diclofenac, carboxyfluorescein) was maybe also due to the lower TEER in the study with 0.1 % DMSO compared to the study with 1% DMSO. However, the differences were statistically not significant.

Type of study	**TEER (Ω*cm²)**
0.1 % DMSO	117.6 ± 2.42
incl. Celecoxib	121.8 ± 2.42
without serum	123.3 ± 2.42
minus Fib medium	124.6 ± 2.42
with Verapamil	127.4 ± 6.42
without CF	127.4 ± 4.85
without blockers	128.8 ± 6.42
with Probenecid	133.0 ± 2.42
1 % DMSO	134.4 ± 2.42

Table 4.14 TEER values
The table shows the TEER values of each group study measured on the day before each experiment.

Group studies/Time interval: 40-240 min.

Date & Study	Substance	Ratio PE_{all}/CF	Ratio PE_{cell}/CF	Ratio PE_{all}/DA	Ratio PE_{cell}/DA	Ratio $PE_{all}DA/CF$	Ratio $PE_{cell}DA/CF$
3/2/2009 **1% DMSO**	Piroxicam	0.41 ± 0.03	0.28 ± 0.03	1.13 ± 0.01	1.39 ± 0.05	2.78 ± 0.24	4.97 ± 0.70
	Ibuprofen	0.50 ± 0.03	0.34 ± 0.03	1.38 ± 0.04	1.68 ± 0.10		
	Meloxicam	0.57 ± 0.04	0.45 ± 0.04	1.56 ± 0.03	2.23 ± 0.13		
	Tenoxicam	0.62 ± 0.02	0.49 ± 0.02	1.71 ± 0.08	2.43 ± 0.24		
	Diclofenac	0.69 ± 0.05	0.54 ± 0.06	1.91 ± 0.02	2.65 ± 0.10		
10/2/2009 **0.1% DMSO**	Piroxicam	0.45 ± 0.02	0.32 ± 0.02	1.08 ± 0.01	1.32 ± 0.02	2.41 ± 0.12	4.16 ± 0.24
	Ibuprofen	0.60 ± 0.05	0.46 ± 0.05	1.44 ± 0.06	1.89 ± 0.15		
	Meloxicam	0.64 ± 0.03	0.53 ± 0.03	1.54 ± 0.02	2.20 ± 0.06		
	Tenoxicam	0.66 ± 0.04	0.56 ± 0.04	1.60 ± 0.02	2.33 ± 0.08		
	Diclofenac	0.88 ± 0.03	0.74 ± 0.03	2.13 ± 0.03	3.06 ± 0.07		
17/2/2009 **minus Fib medium**	Piroxicam	0.45 ± 0.08	0.31 ± 0.08	1.54 ± 0.09	2.10 ± 0.22	3.46 ± 0.42	6.90 ± 1.27
	Ibuprofen	0.81 ± 0.10	0.66 ± 0.10	2.78 ± 0.04	4.47 ± 0.19		
	Tenoxicam	0.78 ± 0.13	0.70 ± 0.15	2.66 ± 0.17	4.69 ± 0.42		
	Meloxicam	0.78 ± 0.13	0.70 ± 0.15	2.65 ± 0.17	4.70 ± 0.42		
	Diclofenac	1.08 ± 0.20	0.98 ± 0.24	3.69 ± 0.31	6.55 ± 0.75		
3/3/2009 **without serum**	Ibuprofen	0.56 ± 0.01	0.34 ± 0.02	1.10 ± 0.01	1.31 ± 0.05	1.98 ± 0.06	3.91 ± 0.39
	Diclofenac	0.60 ± 0.01	0.39 ± 0.01	1.19 ± 0.02	1.51 ± 0.09		
	Piroxicam	0.62 ± 0.01	0.41 ± 0.02	1.23 ± 0.01	1.61 ± 0.07		
	Meloxicam	0.70 ± 0.01	0.52 ± 0.004	1.38 ± 0.04	2.04 ± 0.19		
	Tenoxicam	1.09 ± 0.13	0.84 ± 0.18	2.16 ± 0.23	3.26 ± 0.50		
10/3/2009 **without CF**	Piroxicam	-	-	1.16 ± 0.02	1.45 ± 0.04	-	-
	Tenoxicam	-	-	1.50 ± 0.21	2.09 ± 0.48	-	-
	Meloxicam	-	-	1.50 ± 0.01	2.18 ± 0.08	-	-
	Ibuprofen	-	-	1.62 ± 0.07	2.20 ± 0.15	-	-
	Diclofenac	-	-	1.92 ± 0.07	2.75 ± 0.15	-	-

Table 4.15 shows every group transport study ordered according to ratio values DA/substance in the time interval 40-240 minutes.

Group studies/Time interval: 40-240 min.

Date & Study	Substance	Ratio PE_{all}/CF	Ratio PE_{cell}/CF	Ratio PE_{all}/DA	Ratio PE_{cell}/DA	Ratio $PE_{all}DA/CF$	Ratio $PE_{cell}DA/CF$
17/3/2009 **with Celecoxib**	Piroxicam	0.66 ± 0.08	0.44 ± 0.09	1.62 ± 0.19	1.81 ± 0.38	2.47 ± 0.02	4.11 ± 0.21
	Ibuprofen	0.76 ± 0.06	0.51 ± 0.06	1.87 ± 0.13	2.11 ± 0.27		
	Meloxicam	0.66 ± 0.03	0.55 ± 0.03	1.64 ± 0.06	2.26 ± 0.19		
	Tenoxicam	0.66 ± 0.04	0.55 ± 0.04	1.62 ± 0.09	2.28 ± 0.26		
	Diclofenac	0.89 ± 0.08	0.72 ± 0.08	2.20 ± 0.17	2.96 ± 0.41		
	Celecoxib	1.05 ± 0.06	0.84 ± 0.06	2.59 ± 0.13	3.45 ± 0.34		
21/4/2009 **without blockers**	Piroxicam	0.35 ± 0.02	0.20 ± 0.01	1.09 ± 0.02	1.47 ± 0.09	3.15 ± 0.21	7.31 ± 1.15
	Ibuprofen	0.54 ± 0.03	0.33 ± 0.03	1.69 ± 0.04	2.41 ± 0.18		
	Tenoxicam	0.55 ± 0.03	0.40 ± 0.03	1.73 ± 0.02	2.91 ± 0.19		
without	Celecoxib	0.70 ± 0.02	0.40 ± 0.02	2.19 ± 0.10	2.92 ± 0.38		
Melox	Diclofenac	0.65 ± 0.04	0.44 ± 0.04	2.03 ± 0.01	3.22 ± 0.18		
	Meloxicam	-	-	-	-		
24/3/2009 **with Probenecid without Melox**	Piroxicam	0.44 ± 0.02	0.29 ± 0.02	1.29 ± 0.05	1.79 ± 0.13	2.91 ± 0.07	6.15 ± 0.17
	Ibuprofen	0.65 ± 0.08	0.44 ± 0.11	1.89 ± 0.28	2.75 ± 0.74		
	Diclofenac	0.68 ± 0.02	0.49 ± 0.03	1.99 ± 0.10	3.00 ± 0.28		
	Celecoxib	0.81 ± 0.02	0.55 ± 0.02	2.37 ± 0.09	3.39 ± 0.23		
	Tenoxicam	1.45 ± 0.25	1.40 ± 0.31	4.20 ± 0.64	8.55 ± 1.69		
	Meloxicam	-	-	-	-		
31/3/2009 **with Verapamil without Melox**	Piroxicam	0.51 ± 0.02	0.31 ± 0.02	1.37 ± 0.10	1.59 ± 0.24	2.69 ± 0.12	5.12 ± 0.50
	Ibuprofen	0.84 ± 0.11	0.52 ± 0.12	2.28 ± 0.33	2.69 ± 0.77		
	Tenoxicam	0.68 ± 0.01	0.54 ± 0.01	1.82 ± 0.08	2.74 ± 0.27		
	Diclofenac	0.93 ± 0.02	0.68 ± 0.02	2.51 ± 0.13	3.48 ± 0.40		
	Celecoxib	1.09 ± 0.03	0.79 ± 0.03	2.95 ± 0.18	4.06 ± 0.51		
	Meloxicam	-	-	-	-		

Table 4.16 shows every group transport study ordered according to ratio values DA/substance in the time interval 40-240 minutes.

4.2.3 Comparison between time intervals

Generally, we can observe a tendency of substances to accelerate from the first time interval (0-40 min.) until the second one (40-240 min.) expressed by the PE_{cell} values. Changes in the ranking were connected with this fact, but usually it concerned only one, two or three substances from all tested NSAIDs (5-6) and in many cases they only switched the place with the substance in the ranking above. The illustration of variation in the rankings during different time intervals is shown in the following graphs (Figure 4.2 and 4.3) summarising the PE_{cell} values normalised to diazepam. For better interpretation in the graphs reciprocal ratio values were used.

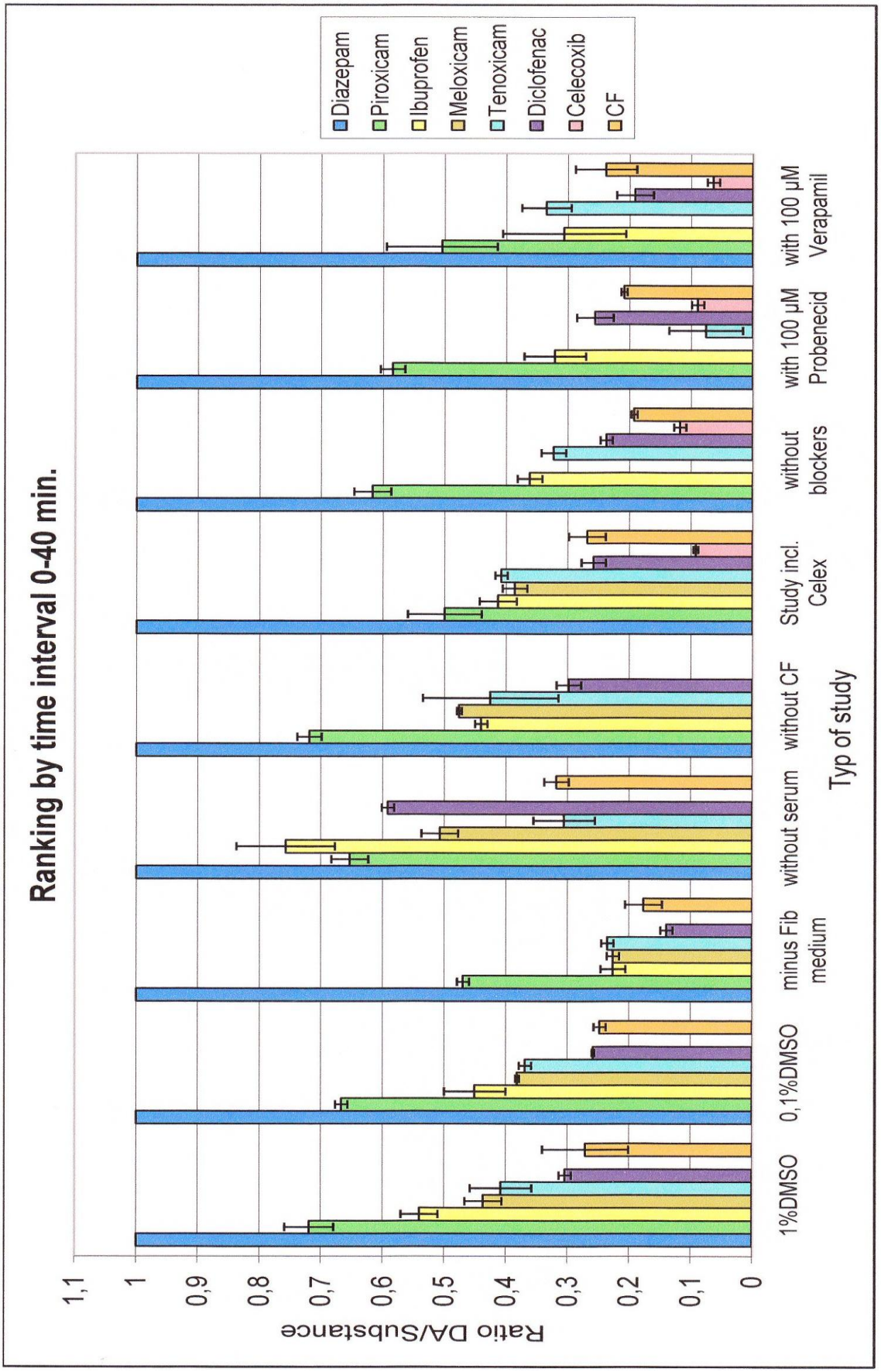

Figure 4.2 Conversed ratio data DA/substance by time interval 0-40 minutes.

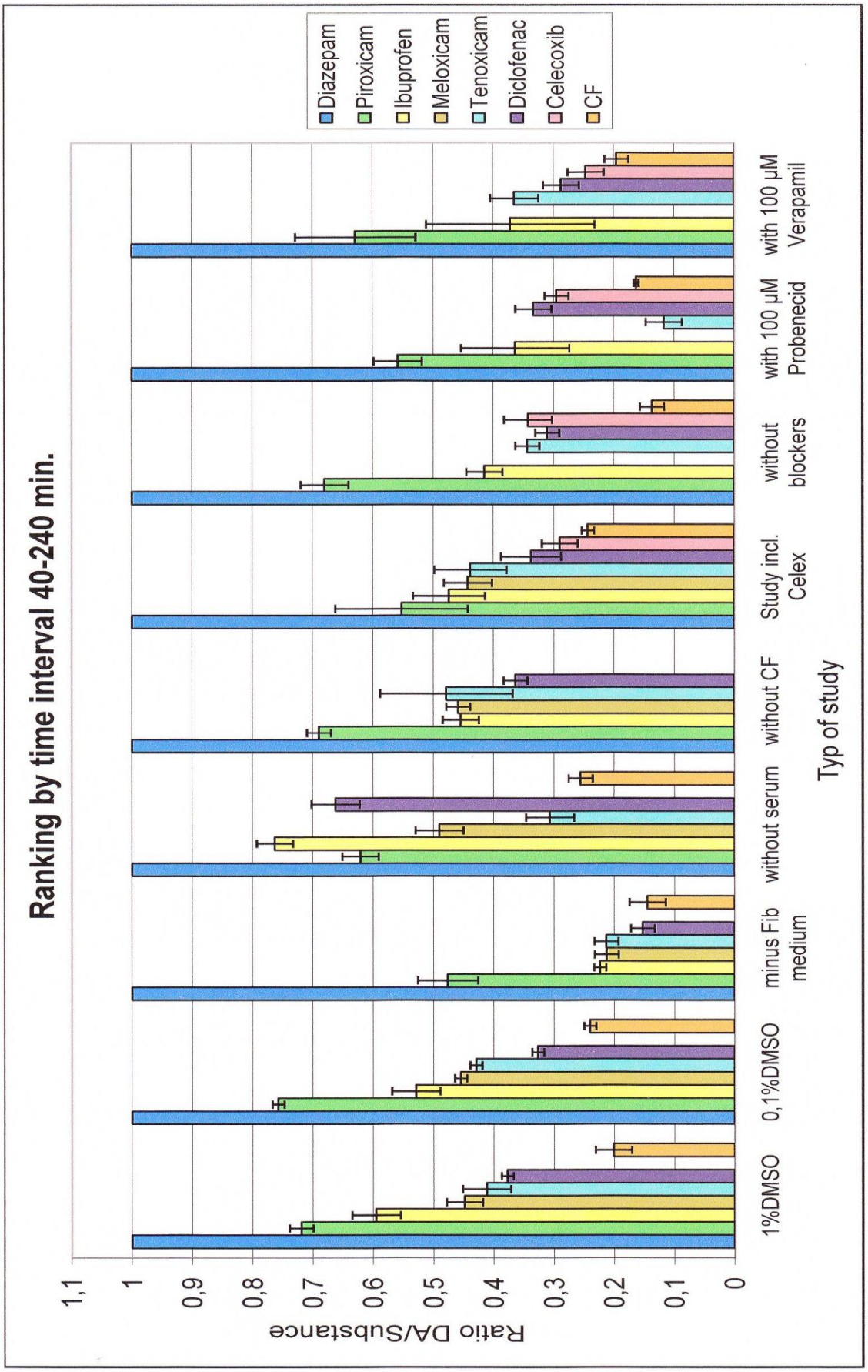

Figure 4.3 Conversed ratio data DA/substance by time interval 40-240 minutes.

4.3 Single studies vs. group study included celecoxib

When we want to compare single and group studies, we have to take the group study, which contained all substances from chosen NSAIDs – ibuprofen, diclofenac, celecoxib, meloxicam, tenoxicam and piroxicam, further internal standards – diazepam and CF and 1 % DMSO. Only this study offered the same conditions as the single studies.

Although the PE_{cell} values by diazepam and CF have been changing by each single study, after all, diazepam was always the fastest substance and CF almost the slowest one. The rankings with regard to different time intervals for both single and group studies are illustrated in the Table 4.17.

Ranking of substances			
Time interval 0-40 minutes		Time interval 40-240 minutes	
Type of study		Type of study	
single	group	single	group
Diazepam	Diazepam	Diazepam	Diazepam
Piroxicam	Piroxicam	Piroxicam	Piroxicam
Ibuprofen	Ibuprofen	Ibuprofen	Ibuprofen
Tenoxicam	Tenoxicam	Tenoxicam	Meloxicam
CF	Meloxicam	Lornoxicam	-
Meloxicam	CF	Meloxicam	Tenoxicam
Lornoxicam	-	Diclofenac	Diclofenac
Diclofenac	Diclofenac	CF	Celecoxib
Celecoxib	Celecoxib	Celecoxib	CF

Table 4.17 Ranking of substances compared by two time intervals and by both types of studies.

At first the comparison between single studies and group study in the time interval 0-40 minutes was assessed. If the presence of lornoxicam in the single studies is not considered, it can be observed that the ranking for the first four substances is the same. A small difference is detected for meloxicam, which was equally fast as CF in the single study (3.61 ± 0.45 to 3.82 ± 1.06 μm/min), but was then faster than CF in the group study (6.93 ± 0.58 to 4.87 ± 0.75 μm/min). Diclofenac and celecoxib were during both types of study slower than CF.

On the other hand, the second time interval (40-240 min.) shows also some changes. The top of the ranking is occupied by diazepam, piroxicam and ibuprofen in both, single and group studies. Further, tenoxicam was little faster than meloxicam (7.40 ± 0.10 to 6.10 ± 0.65 μm/min) in the single studies compared to the group study, where their permeability coefficients overlapped (9.97 ± 0.46 to 10.05 ± 0.63 μm/min). Diclofenac is in the same position in both studies. Finally, CF and celecoxib switched the last two positions comparing single to group studies.

Generally, diazepam, piroxicam and ibuprofen were always on the top positions and in the same ranking. Tenoxicam and meloxicam had a tendency to stay in the middle of the ranking. Concerning CF, it was evident that its permeability ranking decreased with time in both single and group studies. However, considering the PE_{cell} values of CF, which were more or less similar, it was obvious that the permeability of the other substances (meloxicam, diclofenac and celecoxib) increased.

5 DISCUSSION

It was observed in patients using ibuprofen as long-term treatment of rheumatoid arthritis that they suffer less from Alzheimer‘s disease in comparison to persons with the same age without any need to use ibuprofen (or any other NSAIDs) for longer time (Dokmeci, 2004). That pointed to the fact that ibuprofen is passing the BBB and is reducing the formation of beta-amyloid plaques in the brain (which are connected with the genesis of Alzheimer‘s disease). This finding leads to an idea to investigate the conditions under which and how is ibuprofen passing the BBB in more detail.

Considering some studies about ibuprofen the question arose if other NSAIDs could have the same potential. This was supported by the increasing use of NSAIDs during long-term treatment of diseases. Up to now, no systematical compact study including more than three substances to prove permeation abilities of NSAIDs across the BBB and to compare them in single and group studies was made.

In this context, the aim of this diploma thesis was to investigate and compare the abilities of NSAIDs to overcome the BBB. These studies involved seven different NSAIDs (celecoxib, diclofenac, ibuprofen, lornoxicam, meloxicam, piroxicam and tenoxicam), which were tested in single studies as well as in group studies (except lornoxicam) under different conditions.

Single study results revealed following ranking according to the PE_{cell} values (40-240 min.):

diazepam → piroxicam → ibuprofen → tenoxicam → lornoxicam ⟶ meloxicam → diclofenac → carboxyfluorescein (CF) ⟶ celecoxib

As expected, the internal standard diazepam was the fastest followed by piroxicam, ibuprofen, the oxicams (tenoxicam, lornoxicam, meloxicam), diclofenac, internal standard carboxyfluorescein and celecoxib. Surprisingly, celecoxib seemed to be slower than carboxyfluorescein, which was used as a marker of the paracellular transport route. Due to this, two questions arose: 1) Is the transport of celecoxib restricted by an efflux system and 2) Is carboxyfluorescein maybe a substrate of an active transporter in the *in vitro* model used? In order to compare the rankings within the single substance studies, the PE_{cell} values were also normalised to the appropriate PE_{cell} values of diazepam. This resulted in a similar ranking:

piroxicam → ibuprofen → tenoxicam → lornoxicam → celecoxib → meloxicam → diclofenac

Here, celecoxib would have been faster than meloxicam and diclofenac. In this case, the permeability of diazepam was significantly lower than in the other single studies, which may also result from unknown effect. Furthermore, our obtained data from the single substance studies were confronted with a study using epithelial tumour cell line Caco-2, where ibuprofen, piroxicam, diclofenac and meloxicam were investigated (Yazdanian et al., 2004).

This study was comparing permeability coefficients of the permeation of the substances in two directions. The first direction was from the apical part to the basolateral part, where the tested solution was placed into the insert and samples were collected from the wells. Conversely, for studies in the direction basolateral to apical the tested solution was given in the wells and samples were collected from the inserts. The purpose of the study was to compare these two PE values and based on the a-b/b-a ratios to express a possible affinity of substance to an efflux system as e.g. P-glycoprotein. It is known that P-gp is located only on one side of the cell membrane – on the apical side. This knowledge leads to a fact, that if the substance has an affinity to the efflux system, the PE values will be significantly higher in the direction from basolateral to apical. Exactly this was shown for ibuprofen (Apical-basolateral 10.1; Basolateral-apical 19.8). Thus, the ratio of ibuprofen is about 2.0, which may point to a possible interaction with an efflux transport system. Although the ratio was not that significant (compared to other tested substances, but not belonging to the group of NSAIDs), it was the highest from all NSAIDs substances. The ranking in the direction apical to basolateral was:

piroxicam → diclofenac → meloxicam → ibuprofen

The ranking due to basolateral – apical direction was:

diclofenac → ibuprofen → piroxicam → meloxicam

For us the first ranking was important, because our experiments were carried out only in the direction apical to basolateral. Piroxicam was shown as the fastest substance from all NSAIDs, which corresponded with our findings very well. Furthermore, because the ratio of piroxicam was around 1, there was no suspicion on interaction with an efflux system as e.g. P-gp. In our single studies ibuprofen was the second fastest, in the Caco-2 studies ibuprofen was the slowest. From this it could be concluded that ibuprofen can actively be transported and that the transporter machinery is significantly different

between our used ECV304 model compared to the Caco-2 model used by Yazdanian and colleagues (2004). Concerning the single studies with ECV304 cells, interestingly the carboxyfluorescein (CF) values between 0-40 min. were significantly altered during the experiments with diclofenac and celecoxib. The values for CF were the highest in the study with celecoxib, which could be interpreted as a hint for the disruption of the barrier by celecoxib. In addition, permeability of celecoxib increased steadily with time in this study but the PE_{cell} of CF did not. Thus, a disruption of the paracellular barrier by celecoxib should be excluded, it is more probable that celecoxib influenced the functionality of the transporter system of the cell layers. With regard to the study with diclofenac, CF values were quite low. Considering these first results, it was proposed that at least ibuprofen, celecoxib, carboxyfluorescein and possible diclofenac are substrates of transporters, which are perhaps also present and active in our BBB model. Thus, it was decided to accomplish group studies in which the NSAIDs will permeate across the cell layers simultaneously. This enables direct comparison of the permeability rankings and to investigate the influence of different parameters on the NSAIDs permeability at the same time across the same cell layers. Recently, Neuhaus et al. (2008b) showed that group studies revealed an increased reproducibility of permeability data. However, the possible influence of one substance on the permeability of another has to be considered.

The first group studies were with 1% and 0.1 % DMSO in order to compare the influence of the organic solvent on the permeability of the substances. No differences in the permeabilities were observed thus legitimating the usage of 1 % DMSO for all further studies. The small, but not significant differences of diclofenac and ibuprofen are probably related to the different TEER values during these studies enabling an increase of the paracellular transport of these substances which are mainly negatively charged under the experimental conditions (pH = 7.4) and thus also use the paracellular transport route partly. However, the influence of the organic solvent should not be underestimated. Earlier studies of Neuhaus (2007) with the same substances except CF with total amounts of organic solvents of 3.5 % methanol and 1 % acetonitril but no DMSO revealed significant differences. The ranking there was as follows:

diazepam → ibuprofen → piroxicam = diclofenac → tenoxicam → meloxicam

In other studies the influence of DMSO on the permeability frame between CF and diazepam was confirmed (data not shown). Reflecting these results, it is recommended to

use no DMSO, if it is possible. This is very dependent on the solubilities of the used substances and often it is hardly possible to work without DMSO. However, no difference between 1 % and 0.1 % DMSO was shown, thus 1 % DMSO was used in the further group studies.

The next group study was carried out in PBMEC minus Fib medium, which still contained astrocytic factors. This ACM containing medium was also used to cultivate the cells on the inserts and to induce and maintain BBB properties. Comparison of the PE_{cell} values to the 1 % DMSO study revealed significant effects mainly on ibuprofen and slight effect on diclofenac, meloxicam and tenoxicam. In addition, the difference between the internal marker diazepam and the other substances increased indicating a bigger permeability frame in which the different substances can be distinguished. In conclusion, it could be recommended to accomplish the experiments in ACM containing medium since it is assumed that important BBB properties remained to a greater extent than in the C6 medium.

The study without serum showed that the protein binding during the studies is also a very important factor. The permeability of all substances increased in comparison to the 1 % DMSO study due to their higher free fractions. Interestingly, also the rankings changed and ibuprofen and diclofenac became the fastest NSAIDs. Recent studies reported that brain permeability of ibuprofen was dependent on the free plasma amount of ibuprofen (Parepally et al., 2006). The transport rate was quite high, but decreased significantly, when albumin concentration was increased. Furthermore, ibuprofen transport was saturable and inhibited by indometacin. These data suggested a specific transport system for ibuprofen. Typical transport inhibitors as probenecid, pyruvate, digoxin and valproate did not affect the transport rates. Consequently, participation of several transporters as MCT1 (pyruvate), OAT3 (probenecid), oatps 1a4, 1a5 or 1c1 (probenecid and/or digoxin), MRP4 and 5 (probenecid) and valproate transporters were excluded (Parepally et al., 2006). In this context, group studies with probenecid and verapamil showed no significant changes of the ratio normalised to diazepam for ibuprofen. Our data completely confirmed the results that no effect was found with probenecid for ibuprofen and the influence of the serum was proven. However, studies with probenecid resulted in a changed ranking. Tenoxicam decreased remarkably suggesting that tenoxicam interacts with probenecid and is maybe a substrate of one, which also transports probenecid. With regard to the study without CF, it seemed that ibuprofen and diclofenac may interact with CF. CF was reported to be a

substrate for MRP2 and OAT1 (Cantz et al., 2000; Cihlar et al., 2000), thus ibuprofen and diclofenac are also possible MRP2 or OAT1 substrates. However, the presence and functionality of these transporters in our used cell line ECV304 has to be elucidated in further studies. The study with verapamil suggested celecoxib as a substrate for a verapamil dependent transporter. This could be P-gp but also an H^+/tert. amine transporter. Celecoxib is known to down-regulate P-gp function by the inhibition of COX-2 signalling, but not to act as a P-gp substrate. Since very low amounts of P-gp in cell line ECV304 were reported and celecoxib also possesses an amine group, it is worth to investigate whether celecoxib is interacting with an amine transporter, which can also be blocked by verapamil.

In summary, several observations and recommendations could be made based on the results of this thesis:

1. Usage of medium containing astrocytic factors is recommended in order to maintain BBB functions during the experiments.
2. The amount of DMSO (1 % or 0.1 %) did not influence the permeability data. However, DMSO is known as a permeation enhancer and the usage of it should be avoided if possible.
3. The amount of serum can influence the permeability data by increased free fractions of the substances, BUT possibly also by influencing the transporter machinery through e.g. the present growth factors.
4. Carboxyfluorescein is widely used as paracellular marker. However, studies showed its influence on the transport of substances with a carboxylic group as ibuprofen and diclofenac. Thus, CF as a MRP or OAT substrate should not be used as paracellular transport marker since the influence of MRPs or OAT cannot be excluded or distinguished from the paracellular part.
5. Ibuprofen, diclofenac, CF, tenoxicam and celecoxib are supposed to be substrates of transporters at the used BBB model.
6. Meloxicam is possibly also a transporter substrate since its absence increased the ratios to diazepam for tenoxicam. Thus, studies with meloxicam and probenecid would enlighten this issue.

6 CONCLUSION

The purpose of the whole study was to find out as many information as possible about the permeability properties of chosen NSAIDs across a blood-brain barrier *in vitro* model. Because two different types of studies - single and group - were carried out, many data were obtained which then were compared and used to draw conclusions.

As it noticed in almost all studies - single and group - piroxicam permeated the barrier as the fastest substance from all chosen NSAIDs. This primacy of piroxicam was disturbed only in one group study with the medium without serum, where ibuprofen was faster. Probably a stable higher free fraction of piroxicam evokes its higher permeation ability.

Another interest awoke for celecoxib and its ability to accelerate so significantly after the first 40 minutes. This could be connected to its toxicity or that the cell layer adopted its transporter systems for celecoxib.

Anyway, it should still be in mind that our obtained data are not definitive for the human *in vivo* situation, because the transwell model presents just one possibility how to observe the permeation abilities of substances. In addition to other cell lines or primary cells, which could be use, the transwell model presents a static model, which does not include any shear stress. Since endothelial cells and the whole BBB in the human body are exposed to shear stress all the time and it was shown that shear stress can modulate BBB properties, further investigations by using other models (e.g. flow-based hollow-fibre models) and extending the conditions could elucidate the relation of NSAIDs to transport mechanisms.

In summary, it was shown that the ranking of the permeabilities of the NSAID could alter dependent on the experimental conditions as serum content, group compositions, presence of astrocytic factors or transport inhibitors. With regard to the results, it is very likely that active transporters were present in the model used for ibuprofen, diclofenac, carboxyfluorescein, tenoxicam and celecoxib. Thus, future studies may focus on studying the reproducibility of these effects in other models and to elucidate the transport mechanisms of the mentioned NSAIDs in more detail by e.g. concentration-dependent, temperature-dependent, pH-dependent and inhibitor dependent experiments.

7 REFERENCES

Abbott, N. J. (2005a): Dynamics of CNC Barriers: Evolution, Differentiation, and Modulation, Cellular and Molecular Neurobiology. 25: 5-23

Abbott, N.J. (2005b): Physiology of the blood-brain barrier and its consequences for drug transport to the brain. ICS 1277: 3-18

Abbott, N. J., Rönnbäck, L. et al. (2006): Astrocyte–endothelial interactions at the blood–brain barrier. Nat Rev Neurosci. 7: 41-53

Balabanov, R., Dore-Dufy, P. (1998): Role of the CNS microvascular pericyte in the blood-brain barrier. J.Neurosci. Res. 53: 637-644

Ballabh, P., Braun, A., and Nedergaard, M. (2004): The blood-brain barrier: an overview. Structure, Regulation, and clinical implications. Neurology of Disease 16. 1-13

Beckton and Dickinson Company: BD Biosciences. http://www.bdbiosciences.com/discovery_labware/technical_resources/cellculture.shtml accessed on 15.7. 2009

Burian, M., Geisslinger, G. (2005): COX-dependent mechanisms involved in the antinociceptive action of NSAIDs at central and peripheral sites. Pharmacology & Therapeutics 107: 139-154

Cantz, T., Nies, A.T., Brom, M., Hofmann, A.F., Keppler, D. (2000): MRP2, a human conjugate export pump, is present and transports fluo 3 into apical vacuoles of Hep G2 cells Am. J. Physiol. Gastrointest. Liver Physiol. 278: G522-G531

Cecchelli, R., Berezowski, V., Lundquist, S., Culot, M., Renftel, M., Dehouck, MP., Fenart, F. (2007): Modelling of the blood–brain barrier in drug discovery and development Nat Rev Drug Discovery 6: 650-661

Cihlar, T., Ho, E.S. (2000): Fluorescence-based assay for the interaction of small molecules with the human renal organic anion transporter 1 Anal. Biochem. 283: 49-55

Dokmeci, D. (2004): Ibuprofen and Alzheimer´s disease. Folia Med (Plovdiv) 46: 5-10

Garavito, R.M., Malkowski, M.G., and de Witt, D. (2002): The structures of prostaglandin endoperoxide H synthases-1 and -2. Prostaglandins Other Lipid Mediat 68-69: 129-152

Giannini, A.J., Houser, J., Giannini, M.C., Loiselle, R.H. (1984): Antimanic effects of verapamil. American Journal of Psychiatry 141(12): 1602–1605

Giannini, A.J., Nakoneczie, A.M., Melemis, S.M., Ventresco, J., Condon, M. (2000): Magnesium oxide augmentation of verapamil maintenance therapy in mania. Psychiatry Research 93: 83–87

Guentert, T.W., Heintz, R.C., Joly, R. (1987): Overview on the pharmacokinetics of tenoxicam. Eur J Rheumatol Inflamm 9(2): 15-25. Review.

Hirschi, K.K., D´Amore, P.A. (1997): Control of angiogenesis by the pericyte: molecular mechanisms and significance. EXS 79: 419-428

Hla, T., and Neilson, K. (1992): Human cyclooxygenase-2 cDNA. Proc. Natl. Acad. Sci. U S A 89: 7384-7388

Huber, J.D., Egleton, R.D., Davis, T, (2001): Molecular physiology and pathophysiology of tight junctions in the blood-brain barrier. Trends Neurosci. 24: 719-725

Hurst, R.D., Fritz, I.B. (1996): Properties of an immortalised vascular endothelial/glioma cell co-culture model of the blood-brain barrier. J. Cell. Physiol. 167(1): 81-88

Joó, F., and Karmushina, I. (1973): A procedure for the isolation of capillaries from rat brain. Cytobios 8: 41-48

Kuchler-Bopp, S., Delaunoy, J.P., Artault, J.C., Zaepfel, M., Dietrich, J.B. (1999): Astrocytes induce several blood-brain barrier properties in non-neural endothelial cells. Neuroreport 26; 10(6): 1347-53

Liebner, S., Fischmann, A., Rascher, G., Duffner, F., Grote, E.H., Kalbacher, H., Wolburg, H. (2000a): Caludin-1 and claudin -5 expression and tight junction morphology are altered in blood vessels of human glioblastoma multiforme. Acta Neuropathol. (Berl.) 100: 323-331

Liebner, S., Kniesel, U., Kalbacher, H., Wolburg, H., (2000b): Correlation of tight junction morphology with the expression of tight junction proteins in blood-brain barrier endothelial cells. Eur. J. Cell Biol. 79: 707-717

Lincová, D., Farghali, H. et al. (2007): Základní a aplikovaná farmakologie. ISBN: 978-80-7262-373-0

Neuhaus, W., Lauer, R., Oelzant, S., Fringeli, U.P., Ecker, G.F, Noe, C.R. (2006): A novel flow based hollow-fiber blood–brain barrier in vitro model with immortalised cell line PBMEC/C1–2. J Biotechnol. 20; 125(1): 127-41.

Neuhaus, W. (2007): Development and validation of in vitro models of the blood-brain barrier. Dissertation. Department of Medicinal Chemistry, University of Vienna.

Neuhaus, W., Trauner, G., Gruber, D., Oelzant, S., Klepal, W., Kopp, B., Noe, C.R. (2008a): Transport of a $GABA_a$ receptor modulator and ist derivatives from Valeriana officinalis L.s.l. across an in vitro cell culture model of the blood-brain barrier. Planta Med. 74(11): 1338-1344

Neuhaus, W., Wirth, M., Plattner, V.E., Germann, B.. Gabor, F., Noe, C.R. (2008b): Expression of Claudin-1. Claudin-3 and Claudin-5 in human blood-brain barrier mimicking cell line ECV304 is inducible by glioma-conditioned media. Neurosci Lett.3. 446(2-3): 59-64

O´Kane, R.L., et al. (1999): Na^+ -dependent glutamate transporters (EAAT1, EAAT2, and EAAT3) of the blood-brain barrier. J.Biol.Chem. 274: 31891-31895

Olkkola, K.T., Brunetto, A.V., Mattila, M.J. (1994): Pharmacokinetics of oxicam nonsteroidal anti-inflammatory agents. Clin Pharmacokinet 26(2): 107-20. Review

Pardridge, W.M. (2005): Grug and gene targeting to the brain via blood-brain barrier receptor-mediated transport system. ICS 1277: 49-62

Parepally, J.M., Mandula, H., Smith, Q.R. (2006): Brain uptake of nonsteroidal antiinflammatory drugs: ibuprofen, flurbiprofen, and indomethacin. Pharm. Res. 23: 873-881

Steinhilber, D., Schubert-Zsilavecz, M., Roth, H.J. (2005): Medizinische Chemie: Targets und Arzneistoffe. Deutscher Apotheker Verlag. Stuttgart. Germany. Ramsauer, M., Krause, D., Dermietzel, R. (2002): Angiogenesis of the blood-brain barrier in vitro and the functiona of cerebral pericytes. FASEB J. 16: 1274-1276

Szkokan, P. (2009): Entwicklung eines *in-vitro* Infektionsmodells der Blut-Hirn Schranke (Diploma thesis)

Ribatti, D., Nico, B., Crivellato, E., Artico, M. (2006): Development of the blood-brain barrier: a historical point of view. Anat Rec B New Anat. 289(1): 3-8

Vane, J.R. (1971): Inhibition of prostaglandin sysnthesis as a mechanism of action for aspirin-likw drugs. Nat.New. Biol. 231(25): 232-235

Yazdanian, M., Briggs, K.., Jankovsky, C., and Hawi, A. (2004): The „High Solubility“ Definitio of the Current FDA Guidance on Biopharmaceutical Classification System May Be Too Strict for Acidic Drugs. Pharmaceutical Research 21: 293-299

Zarghi, A., Shafaati, A., Foroutan, S.M., Khoddam, A. (2006): Simple and rapid high-performance liquid chromatographic method for determination of celecoxib in plasma UV detection: Application in pharmacokinetic studies. J.of Chromatography B 835: 100-104