ANNE MANNILA

Central Nervous System Permeation of Ibuprofen, Ketoprofen and Indomethacin

In Vivo and *In Situ* Studies in Rats and Clinical Trials in Children

Doctoral dissertation

To be presented by permission of the Faculty of Pharmacy of the University of Kuopio for public examination in Auditorium, Mediteknia building, University of Kuopio, on Friday 22th May 2009, at 12 noon

Department of Pharmaceutical Chemistry
Faculty of Pharmacy
University of Kuopio

KUOPION YLIOPISTO

KUOPIO 2009

Author's address: Department of Pharmaceutical Chemistry
                    University of Kuopio
                    P.O. Box 1627
                    FI-70211 KUOPIO
                    FINLAND

                    Current address:
                    Centre for Drug Candidate Optimisation
                    Monash Institute of Pharmaceutical Sciences
                    Monash University
                    381 Royal Parade, Parkville VIC 3052, Australia
                    E-mail: anne.mannila@pharm.monash.edu.au

ABSTRACT

Many compounds have a limited ability to penetrate into the central nervous system (CNS) due to the existence of sophisticated barrier systems between the CNS and blood. The blood-brain barrier (BBB) between cerebral blood and the interstitial fluid of the brain, and the blood-cerebrospinal fluid barrier (BCSFB) between blood and ventricular and subarachnoid cerebrospinal fluid (CSF), both act as physical, metabolic and efflux barriers. In addition, they express several influx transporters and receptors. As a consequence, the BBB and the BCSFB can regulate extremely efficiently the CNS transport of both small and large molecules. The CNS distribution of non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen, indomethacin and ketoprofen is of interest, because in addition to their well-known peripheral effects, they may also have important central actions. For example in children, the central antinociceptive actions of these widely used NSAIDs rationalizes the need for understanding their CNS distribution. Furthermore, in adults and the elderly, the long-term use of NSAIDs may reduce the risk, or delay the onset of Alzheimer's disease (AD). In order to act at the central level, a sufficient amount of a drug must gain access to the CNS. NSAIDs are weak acids and are ionized at the pH of the circulation and additionally, they are extensively bound to plasma proteins; two characteristics which may limit their CNS permeation.

In the present study, the brain permeation of ibuprofen and indomethacin was determined in rats after intravenous infusion. In addition, the effect of plasma protein binding on the brain permeation of indomethacin was studied with the *in situ* brain perfusion technique. The effect of an efflux inhibitor, probenecid, on brain permeation of indomethacin was evaluated both after intravenous administration, and after *in situ* brain perfusion method. The CSF distribution of ketoprofen and indomethacin was evaluated in children, aged 4-144 months. Children were given ketoprofen or indomethacin intravenously prior to surgery with spinal anaesthesia. Simultaneous venous blood and CSF samples were collected once from each child 7-67 minutes after ketoprofen administration and 14-225 minutes after indomethacin administration. Drug concentrations were determined in CSF, plasma and protein-free plasma, and the concentration ratio between CSF and plasma was calculated.

Brain penetration of both ibuprofen and indomethacin was found to be low in rats, the total brain to plasma ratio being less than 0.02. Co-administration of probenecid increased the brain to plasma concentration ratio of indomethacin by 2.4-fold. Co-administration of probenecid increased also the unbound indomethacin concentration by 6.3-fold. The single point unidirectional transfer constant (K_{in}) of indomethacin was 2.2 ± 0.2 x 10^{-3} ml/s/g brain. The K_{in} value of indomethacin in the presence of probenecid was 3.7 ± 0.3 x 10^{-3} ml/s/g brain, and 1.9 ± 0.3 x 10^{-3} and 0.5 ± 0.1 x 10^{-3} ml/s/g brain after perfusion with a perfusion fluid containing 0.28% and 2.8 % (w/v) bovine serum albumin, respectively. In children, the CSF to total plasma concentration ratios of both ketoprofen and indomethacin remained less than 0.01 at all times. The ketoprofen concentration in the CSF ranged from 1.4 to 24 ng/ml (median 6.6 ng/ml) after the dose of 1 mg/kg. The indomethacin concentrations in the CSF ranged from 0.2 to 5.0 ng/ml (median 1.4 ng/ml) after a dose of 0.35 mg/kg.

In conclusion, ibuprofen and indomethacin permeated poorly into the rat brain after intravenous administration. The efflux protein inhibitor, probenecid, was able to increase the brain permeation of indomethacin. The increase in the unbound fraction after co-administration of probenecid may explain the enhanced brain permeation of indomethacin after intravenous administration. However, some effect on the efflux systems at the BBB cannot be ruled out as co-administration of probenecid slightly increased the initial brain uptake of indomethacin from protein-free medium. Ketoprofen and indomethacin are able to permeate the CSF of children after intravenous administration. The CSF permeation of ketoprofen and indomethacin was limited after intravenous administration since only less than 1% of the total plasma drug concentration was found in the CSF. Whether this is sufficient to mediate any central antinociceptive effects in the lumbar space, or be involved in other central mechanisms of action remains to be clarified.

ACKNOWLEDGEMENTS

ABBREVIATIONS

$A\beta$	Amyloid β
AD	Alzheimer's disease
AMT	Absorptive-mediated transcytosis
AUC	Area under the concentration - time curve
BBB	Blood-brain barrier
BBB PS_u	Blood-brain barrier permeability-surface area product to free drug
BCRP	Breast cancer resistant protein
BCSFB	Blood-cerebrospinal fluid barrier
BSA	Bovine serum albumin
BUI	Brain uptake index
CL_{in}	Influx clearance
CL_{out}	Efflux clearance
C_{max}	Maximum drug concentration
CSF	Cerebrospinal fluid
CNS	Central nervous system
COX	Cyclooxygenase
CVO	Circumventricular organ
ECF	Extracellular fluid
GC-MS	Gas chromatography - mass spectrometry
HPLC	High performance liquid chromatography
i.m.	Intramuscular
i.p.	Intraperitoneal
i.v.	Intravenous
ISF	Interstitial fluid
K_{in}	The single point unidirectional transfer constant
LAT1	Large neutral amino acid transporter
Log P	Logarithmic 1-octanol/aqueous phase partition coefficient
MCT	Monocarboxylic acid transporter
MRI	Magnetic resonance imaging
MRP	Multidrug resistant-associated protein
NMDA	N-methyl-D-aspartic acid
NSAID	Non-steroidal anti-inflammatory drug
OAT	Organic anion transporter
OATP	Organic anion transporting polypeptide
OCTN	Organic cation/carnitine transporter
PA	Cerebrovascular permeability-surface area product
PET	Positron emission tomography
P-gp	P-glycoprotein
pKa	Negative logarithm of the ionization constant

RMT	Receptor-mediated transport
RP-HPLC	Reversed phase high performance liquid chromatography
SD	Standard deviation
SEM	Standard error of mean
SPE	Solid phase extraction
SPECT	Single photon computed tomography
SS	Steady-state
t_{max}	Time at which the maximum drug concentration is reached
UDP	Uridine diphosphate

LIST OF ORIGINAL PUBLICATIONS

This work is based on the following publications:

I Anne Mannila, Jarkko Rautio, Marko Lehtonen, Tomi Järvinen, Jouko Savolainen: Inefficient central nervous system delivery limits the use of ibuprofen in neurodegenerative diseases. European Journal of Pharmaceutical Sciences 24: 101–105, 2005

II Anne Mannila, Mikko Gynther, Marko Lehtonen, Anne Lecklin, Tomi Järvinen, Jarkko Rautio, Jouko Savolainen: Effect of an efflux inhibitor probenecid and plasma protein binding on brain permeation of indomethacin in rats. Manuscript

III Anne Mannila, Hannu Kokki, Marja Heikkinen, Merja Laisalmi, Marko Lehtonen, Hanna-Leena Louhisto, Tomi Järvinen, Jouko Savolainen: Cerebrospinal fluid distribution of ketoprofen after intravenous administration in young children. Clinical Pharmacokinetics 45: 737-743, 2006

IV Anne Mannila, Elina Kumpulainen, Marko Lehtonen, Marja Heikkinen, Merja Laisalmi, Terhi Salo, Jarkko Rautio, Jouko Savolainen, Hannu Kokki: Plasma and cerebrospinal fluid concentrations of indomethacin in children after intravenous administration. Journal of Clinical Pharmacology 47: 94-100, 2007

CONTENTS

1 INTRODUCTION

Advances in neurosciences and biotechnology have greatly expanded our knowledge about the physiological and pathological features of the central nervous system (CNS). When this knowledge is combined with advances in pharmaceutical sciences, then one would predict that this could lead to an increase in the number of drugs available for the treatment of CNS diseases. However, many CNS disorders still lack efficient drug therapy. CNS drug development must overcome some fundamental challenges, e.g. many novel agents that appear promising *in vitro* fail to evoke activity *in vivo* in preclinical studies. This is often due to the fact that these agents are not able to permeate into their site of action (i.e. the CNS), because they cannot pass across the barriers between the CNS and blood after systemic administration (Pardridge 2003).

The CNS is protected against internal, blood-borne damage that might either disturb its homeostasis or be toxic by the presence of the sophisticated structures called the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB). These barriers act both as physical, metabolic and efflux barriers (Minn *et al.* 2000). The physical barrier is formed by the cells in the blood-CNS-barriers which prevent the entry of polar and high molecular weight molecules. The metabolic barrier results from the activity of a battery of enzyme systems present in the cells of blood-CNS-barriers. Finally, the various efflux protein systems which are able to transport compounds from the CNS to the systemic circulation create the efflux barrier.

The BBB and the BCSFB are extremely efficient at limiting and regulating the exchange of compounds between the CNS and blood. In general terms, there are four mechanisms by which a compound may access the CNS i.e. transcellular passive diffusion, carrier-mediated transport, receptor-mediated transcytosis and adsorptive-mediated transcytosis (Tsuji 2000). Usually small molecular weight compounds utilize passive diffusion or carrier-mediated transport mechanisms whereas large molecules such as proteins take advantage of receptor- or adsorptive-mediated mechanisms. If a drug is to pass the BBB or the BCSFB by passive diffusion, it must possess favourable physicochemical properties, such as adequate lipophilicity (Begley 2004). In contrast, the carrier- or receptor-mediated mechanisms require specific interactions between the drug and the transport protein, and are therefore limited only to drugs which closely resemble the endogenous substrate of the transport protein or receptor.

The blood-CNS barriers can be bypassed by delivering drugs directly into the brain or CSF e.g. via intraventricular, intraparenchymal, intrathecal and also by the nasal route (Begley 2004). However, the transvascular route, i.e. systemic administration of the drug followed by penetration through the BBB or BCSFB, remains the most straightforward way to deliver drugs to the CNS (Pardridge 2003). Therefore, there is an evident need for obtaining

knowledge about the BBB or BCSFB transport properties of compounds. Gaining such information will enable the development of efficient CNS drug delivery technologies which will hopefully lead to an increase in the number of drugs available for the treatment of CNS diseases.

Often novel CNS drugs are large molecular weight compounds such as proteins and genes which have obvious inherent permeation limitations, but also the CNS permeation of many widely used small molecular weight drugs may not be fully understood. For instance, the CNS distribution of non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen, indomethacin and ketoprofen is of interest because in addition to their well-known peripheral actions, they may also have important central actions. For example, some of their antinociceptive actions are believed to occur in the CNS (Daher and Tonussi 2003, Malmberg and Yaksh 1992, Ossipov et al. 2000). In order to act at the central level, a sufficient amount of a drug must gain access to the CNS. However, little is known about the CNS distribution of NSAIDs such as ibuprofen, indomethacin and ketoprofen. In children in particular, the information about the CNS distribution of these commonly used antinociceptive agents is extremely limited. In addition to being potential centrally acting antinociceptive agents, NSAIDs may have other beneficial central actions. Several epidemiological studies have claimed that long-term use of NSAIDs may reduce the risk or delay the onset of Alzheimer's disease (AD) (Szekely et al. 2004, Etminan et al. 2003, Vlad et al. 2008). NSAIDs are weak acids and ionized at the pH of the systemic circulation and additionally, they are extensively bound to plasma protein albumin (Burke et al. 2006). These characteristics of NSAIDs may limit their CNS permeation, and therefore limit their use as centrally acting agents.

The aim of the present study was to evaluate the CNS disposition of NSAIDs ibuprofen, indomethacin and ketoprofen. The extent of CNS permeation was evaluated based on the ratio between the brain and the plasma concentration in rats or based on the ratio between the CSF and the plasma concentration in children.

2 REVIEW OF LITERATURE

2.1 Central nervous system drug delivery

2.1.1 Central nervous system

Basically, CNS is composed of the brain and the spinal cord. However, in terms of the tissue components, the brain volume can be subdivided into four compartments i.e. parenchyma (brain cells), parenchymal interstitial fluid (ISF), CSF and cerebral blood (Davson and Segal 1996). The cells of the brain include both nerve cells (neurons) and glial cells. The ISF is a flowing fluid providing an optimal microenvironment to bath the brain cells and it is mainly secreted at the cerebral capillary wall (Abbott 2004). The other fluid compartment, the CSF, surrounds the brain and the spinal cord. It is secreted from the blood by the choroid plexus, which is located in the lateral, 3rd and 4th ventricles. The CSF flows from the ventricles through the subarachnoid space into different parts of the brain and spinal cord. Ultimately, the CSF drains through arachnoid granulations into the dural sinuses and then to the venous system. The CSF maintains the medium for the CNS and acts both as an elimination pathway and a mechanical cushion for the brain and spinal cord (Rowland *et al*. 1991). The cerebral vasculature density is high and it has been suggested that every neuron is perfused by its own blood vessel (Pardridge 2003). As a result, the transvascular route is a very efficient way to deliver compounds into the CNS.

There are several pathways for compound transfer between the different CNS compartments (Figure 2.1). The mixing between the ISF and the brain parenchyma is limited, as the bulk flow of the ISF is restricted to the white matter and the perivascular space of the brain (Abbott 2004). The ISF and the CSF are in intimate contact with each other and share a similar composition, e.g. a low protein concentration (Davson and Segal 1996). Together the ISF and the CSF make up the extracellular fluid (ECF) of the CNS. The transfer of solutes between the ISF and the CSF has not been exhaustively investigated. From the CNS drug delivery point of view, it appears that differences in drug concentrations between ISF and CSF are common (De Lange and Danhof 2002, Shen *et al*. 2004).
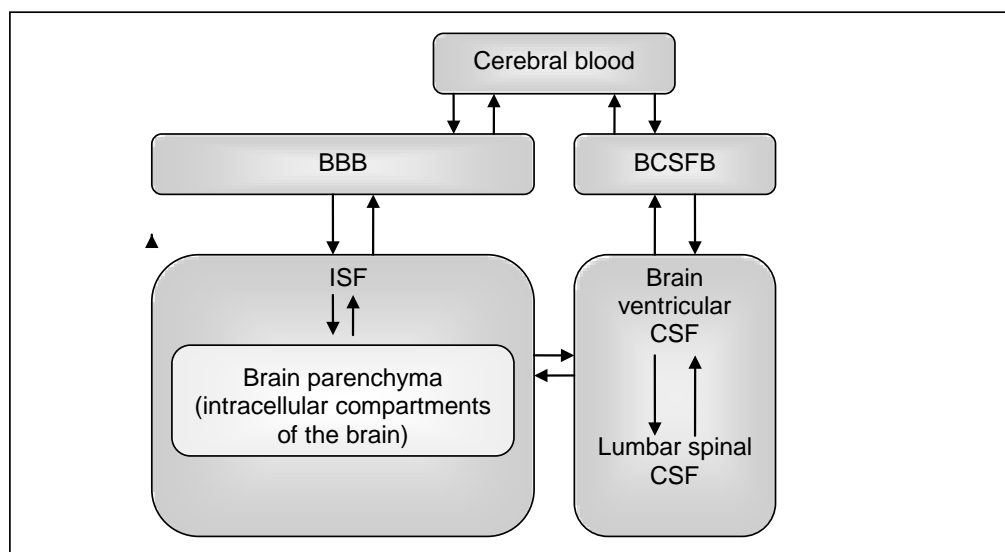
Figure 2.1. Simplified model of the CNS compartments (modified from de Lange and Danhof 2002).

Furthermore, the ISF and the CSF have a role in compound clearance from the CNS. The ISF is a flowing liquid and its clearance occurs primarily by bulk flow along the perivascular space to the CSF (Abbott 2004). Therefore, once a compound is present in the ISF, it can leave the CNS not only by passing through the BBB back to cerebral blood vessels but also through passage into the CSF. The turnover rate of the CSF is faster than that of the ISF (Davson and Segal 1996), and it is recognised that due to its continuous turnover, CSF can act as a sink for brain tissue. Continuous CSF turnover is one of the factors (others including metabolism and active efflux mechanisms in the CNS) that may explain why many compounds have a steady-state ISF or CSF to unbound plasma concentration ratio of less than unity (Table 2.1). The impact of CSF turnover is most pronounced with compounds that penetrate the CNS very slowly, because in these cases the clearance from the CNS by CSF turnover is rapid relative to the influx of the compound across the blood-CNS barriers (Rowland and Tozer 1995, Davson and Segal 1996).

Table 2.1. Examples of compounds that have low CSF or ISF to unbound plasma concentration ratios.

Compound	Dosing	CSF:plasma (Method of collecting CSF)	ISF:plasma	Ref.
Salicylic acid	I.v. infusion in dogs, CSF samples collected 3 hours after reaching SS in plasma	Approximately 0.6 (Cisternal catheter)	-	(Brodie *et al*. 1960)
Sulfaguanidine	I.v. infusion in dogs, CSF samples collected 3 hours after reaching SS in plasma	Approximately 0.4 (Cisternal catheter)	-	(Brodie *et al*. 1960)
Probenecid	SS i.v. infusion in rats	0.629 (Cisternal puncture)	0.199 (Hippocampal microdialysis)	(Deguchi *et al*. 1997)
SDZ EAA 494 (NMDA receptor antagonist)	I.v. injection in rats, comparison of AUCs	0.17 (Cisternal catheter)	0.11 (Frontal cortex microdialysis)	(Amsterdam and Lemaire 1997)
EAB 515 (NMDA receptor antagonist)	SS i.v. infusion in rats	0.18 (Lateral ventricle microdialysis)	0.08 Frontal cortex microdialysis	(Malhotra *et al*. 1994)
Stavudine	SS i.v. infusion in rats	0.50 (Lateral ventricle microdialysis)	0.34 (Frontal cortex microdialysis)	(Yang *et al*. 1997)
Ofloxacin	SS i.v. infusion in rats	0.234 (Cisternal puncture)	0.118 (Transcranial microdialysis)	(Ooie *et al*. 1997)
Fleroxacin	SS i.v. infusion in rats	0.420 (Cisternal puncture)	0.147 (Transcranial microdialysis)	(Ooie *et al*. 1997)
Perfloxacin	SS i.v. infusion in rats	0.369 (Cisternal puncture)	0.147 (Transcranial microdialysis)	(Ooie *et al*. 1997)
Norfloxacin	SS i.v. infusion in rats	0.033 (Cisternal puncture)	0.034 (Transcranial microdialysis)	(Ooie *et al*. 1997)
Baclofen	I.v. injection in rats (Pseudo-SS)	0.0277 (Cisternal puncture)	0.0346 (Hippocampal microdialysis)	(Deguchi *et al*. 1995)
Ganciclovir	SS i.v. infusion in rats	0.067 (Cisternal puncture)	0.073 (Frontal cortex microdialysis)	(Liu *et al*. 2008)

The interaction of the blood compartment with the other compartments is strictly regulated by complex CNS barrier systems. The BBB between cerebral blood and ISF is formed by the cerebrovascular endothelial cells whereas the BCSFB between blood and ventricular and subarachnoid CSF is formed by the epithelial cells in the choroid plexus and arachnoid membrane (Johanson *et al*. 2005). These barriers are crucial in many respects; the CNS can maintain a tight ionic homeostasis, restrict and regulate the turnover of both small and large molecules, separate peripheral and central neurotransmitter pools and maintain immune surveillance (Abbott 2004). The BBB and the BCSFB are crucial barriers to be overcome in successful CNS drug therapy since the systemic route is still considered the most efficient way to deliver drugs into the CNS (Pardridge 2003). Therefore, drugs that cannot pass the BBB or the BCSFB sufficiently cannot be used to treat CNS disorders.

2.1.1.1 The blood-brain barrier

The blood-CNS barriers can be envisaged as a three-part barrier system (Minn *et al*. 2000). The *physical barrier* is formed by the cells in the blood-CNS-barriers and this prevents the entry of polar and high molecular weight molecules into the CNS. The *metabolic barrier* results from the activity of a battery of enzyme systems present in the cells of the blood-CNS-barriers. In addition, various efflux protein systems which are able to remove compounds from the CNS to the systemic circulation create an *efflux barrier.* The physical, metabolic and efflux barriers will be discussed in more detail below.

The physical barrier

The basis of the physical barrier at the level of the BBB is formed by the endothelial cells in the brain capillaries. They are joined together by tight junctions, thus preventing the paracellular transport of compounds. The tight junctions of the brain endothelium are more complex than those present in the peripheral tissue endothelium (Wolburg and Lippoldt 2002). The degree of tightness of these junctions, as measured by transendothelial electrical resistance higher than 1000 Ωcm^2, resembles that found in barrier epithelial cells (Schulze 1996). For comparison, the transendothelial electrical resistance is only about 2-20 Ωcm^2 in peripheral capillaries (Abbott *et al*. 2006). An efficient barrier against paracellular transport is formed not only because of the existence of tight junctions, but also because endothelial cells at the BBB lack fenestrae and because pinocytosis rarely occurs (Schulze 1996).

In addition to the capillary endothelial cells, the BBB contains also pericytes, astrocytes, microglia and nerve endings which contribute to the physical barrier (Abbott *et al*. 2006). For instance, astrocytes form an extra layer around the endothelial cells thus forcing substances to cross several cell membranes in order to pass through the BBB (Figure 2.2).
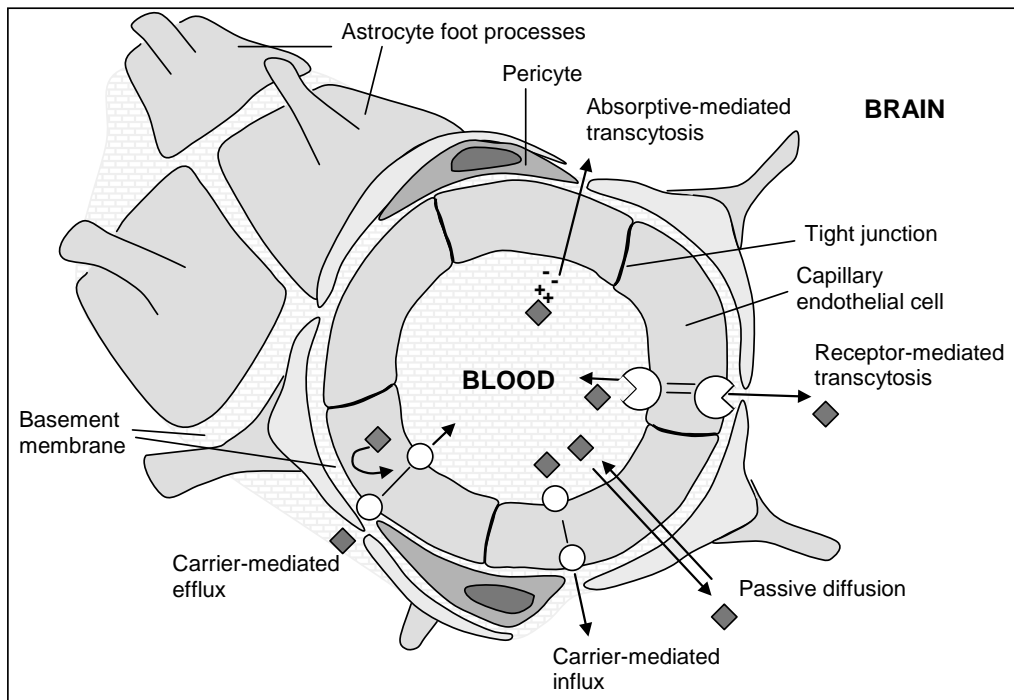
Figure 2.2. Structure and different transport mechanisms present at the BBB.

The metabolic barrier

The metabolic barrier of the BBB consists of various metabolic enzymes. As substances pass the BBB by the transcellular route, they are inevitably exposed to enzymes within the cells at the BBB (Ghersi-Egea *et al*. 1995). The BBB expresses both phase I and phase II enzymes (Minn *et al*. 1991). In addition, ATP-dependent efflux transport mechanisms can be classified as a type of phase III enzymatic system, as they are able to remove polar metabolites from the CNS. Some enzymes, such as γ-glutamyl transferase, alkaline phosphatase, adenosine deaminase and purine-nucleoside phosphorylase have higher activities at the BBB than in the brain tissue (Johnson and Anderson 1996). This provides further evidence that they possess a protective function at the BBB. However, metabolic enzymes have a somewhat contradictory role in the BBB since in addition to their protective functions, their activity can also produce reactive metabolites or oxygen species which may endanger the CNS, e.g. by accelerating neurodegeneration (Minn *et al*. 2000). From a drug delivery point of view, metabolism at the BBB or in the CNS can result in inactivation of pharmacologically active molecules which will impede efficient drug therapy. On the other hand, enzymes located at the BBB can be utilized to achieve site-specific drug delivery to the CNS via a prodrug approach where the probability of success increases if the prodrug bioconversion takes place selectively in the CNS (Anderson *et al*. 1991).

The efflux barrier

P-glycoprotein (P-gp) is probably the most well-known efflux protein which is able to prevent compounds from entering the CNS. It was originally characterized as a protein able to confer multidrug resistance to cancer cells and only later as having a role in CNS distribution and intestinal absorption of drugs (Lin and Yamazaki 2003, Schinkel 1999). P-gp is an ATP-dependent transport protein which is located at the luminal (blood-side) membrane of brain capillary endothelial cells (Schinkel 1999). P-gp is able to recognise and transport a great number of structurally diverse compounds; its substrates include antineoplastic drugs such as doxorubicin, vinblastine and paclitaxel, HIV protease inhibitors nelfinavir, indinavir and saquinavir and the immunosuppressant, cyclosporin A (Schinkel 1999). *In vivo* experiments in P-gp knockout mice and other animal models have established the role played by P-gp in the BBB. For instance, after a single bolus injection, significantly higher brain levels as well as accumulation in the brain tissue of a P-gp substrate digoxin (and the pharmacologically active metabolites of digoxin) were observed in P-gp knockout mice compared to wild-type mice (Mayer *et al*. 1996). Also the plasma levels of digoxin (and its pharmacologically active metabolites) were higher in the P-gp knockout mice indicating the effect of P-gp on the systemic pharmacokinetics of digoxin. The increase in the plasma levels of digoxin explains partly the increased brain levels of digoxin. Other examples showing the functional role of P-gp in the BBB include the study by Wang *et al*. (1995), where increased brain delivery of a P-gp substrate rhodamine-123 was observed in the presence of a P-gp inhibitor cyclosporine A, the study by de Lange *et al*. (1998) where increased brain delivery of rhodamine-123 was observed in P-gp knockout mice compared to wild-type mice, as had also been found by Xie *et al*. (1999) for morphine, another known P-gp substrate. The clinical relevance of P-gp has been demonstrated, for instance in the case of the antidiarrheal compound, loperamide. Loperamide is a potent opiate which does not normally enter the CNS because it is a substrate for P-gp. However, when administered with quinidine, a P-gp inhibitor, loperamide produces respiratory depression indicative of enhanced CNS entry. Thus, potentially dangerous CNS effects can appear due to increased CNS penetration after P-gp inhibition (Sadeque *et al*. 2000).

P-gp is member of the ABC transporter family. Other members of the ABC transporter family that have been suggested to have a role at the BBB include isoforms of multidrug resistance-associated proteins (MRPs) and breast cancer resistant protein (BCRP) (Wijnholds *et al*. 2000, Leggas *et al*. 2004, Cisternino *et al*. 2004). MRP1 and 5, and the BCRP have been suggested to be localised on the luminal side of brain microvessel endothelial cells, whereas MRP4 has been proposed to be situated on both the luminal and abluminal (brain) sides (Zhang *et al*. 2004, Cisternino *et al*. 2004). The substrates for P-gp, MRPs and the BCRP overlap to some extent - MRPs can be characterised as a group of transporters for organic anions, glutathione, or glucuronide-conjugated compounds (Jedlitschky *et al*. 1996), and the BCRP has been reported to transport mitoxantrone, prazosin, anthracyclines and some camphoteric derivatives (Cisternino *et al*. 2004).

Members of the solute carrier families SLC21 and SLC22, i.e. organic anion transporting polypeptides (OATPs), organic anion transporters (OATs) and organic cation/carnitine transporters (OCTNs) are believed to be involved in the transport of a wide range of substrates, such as bile salts, steroid hormones and their conjugates, thyroid hormones, anionic peptides, eicosanoids, cyclic nucleotides, endogenous amines, uric acid, cardiac glycosides, antibiotics, NSAIDs, diuretics, antineoplastic drugs, uricosuric agents, tetraethylammonium and carnitine (Hagenbuch *et al*. 2004, Koepsell and Endou 2004). OATPs, OATs and OCTNs are expressed in many different tissues, and there is evidence for their central role in drug absorption and excretion particularly in intestine, liver and kidney. They are also involved in the efflux of various compounds, particularly organic anions, at the BBB (Hagenbuch *et al*. 2004, Koepsell and Endou 2004, Kusuhara and Sugiyama 2005). There are many members of the OATP family, but at least rat Oatp1a4, Oatp1a5 and Oatp1c1 are expressed in the brain capillaries. Oatp1a4 and Oatp1c1 are thought to be localised both on the luminal and abluminal membranes of the brain capillaries and to be involved in both efflux and uptake of compounds (Kusuhara and Sugiyama 2005). For example, it is suggested that the transport of thyroxine both from the circulating blood to the brain and from brain to blood is mediated by Oatp1c1 (Sugiyama *et al*. 2003). It is also believed that rat Oat3 has a role at the BBB (Kusuhara *et al*. 1999) since it appears to be localised on the abluminal membrane of the brain capillaries and has been shown to be involved in the efflux transport of indoxyl sulphate and thiopurine nucleobase analogs (Mori *et al*. 2004, Kikuchi *et al*. 2003, Ohtsuki *et al*. 2002). From the solute carrier families also monocarboxylic acid transporters (MCTs) may have a role as an efflux mechanism at the BBB, as the CNS distribution of probenecid, salicylate and neotrofin are suggested to be mediated by the MCT system (Deguchi *et al*. 1997, Yan and Taylor 2002).

2.1.1.2 The blood-cerebrospinal fluid barrier

The BCSFB exists at the level of the choroid plexus and the arachnoid membrane (Johanson *et al*. 2005). Traditionally, the choroid plexus has been perceived as having a larger role in compound transfer between blood and the CSF than the arachnoid membrane, and it is thus discussed in more detail. The BCSFB has characteristic structural and functional properties that distinguish it from the BBB. In contrast to the endothelial cells in brain capillaries, the capillaries in the choroid plexus lack tight junctions and are therefore freely permeable. The barrier is formed by the epithelial cells of the choroid plexus which face the CSF (Rowland *et al*. 1991). In the choroid plexus the activity of several metabolic enzymes is relatively high. For example, in the rat choroid plexus the activities of glutathione peroxidase and several cytochrome P450 isoenzymes are high, and the activities of UDP-glucuronosyltransferase and epoxide hydrolase are very high, being at the same level as those in the liver when expressed on a per mg protein basis (Ghersi-Egea *et al*. 1995). Furthermore, at the level of the BCSFB, active transport mechanisms are abundant (Kusuhara and Sugiyama 2004). Although the same

efflux transporters can be found in the choroid plexus and at the BBB (Figure 2.3), functional differences exist between the BCSFB and the BBB (de Lange 2004). For instance, it is believed that the efflux of penicillin occurs specifically at the choroid plexus (Ogawa *et al*. 1994). The role of P-gp at the BCSFB is presently unclear (Kusuhara and Sugiyama 2004). The best-characterised MRP isoform MRP1 is believed to have a role in the efflux of compounds across BCSFB but not to be involved in efflux across the BBB (Wijnholds *et al*. 2000), although not all researchers subscribe to its lack of a role in the BBB (Zhang *et al*. 2004).
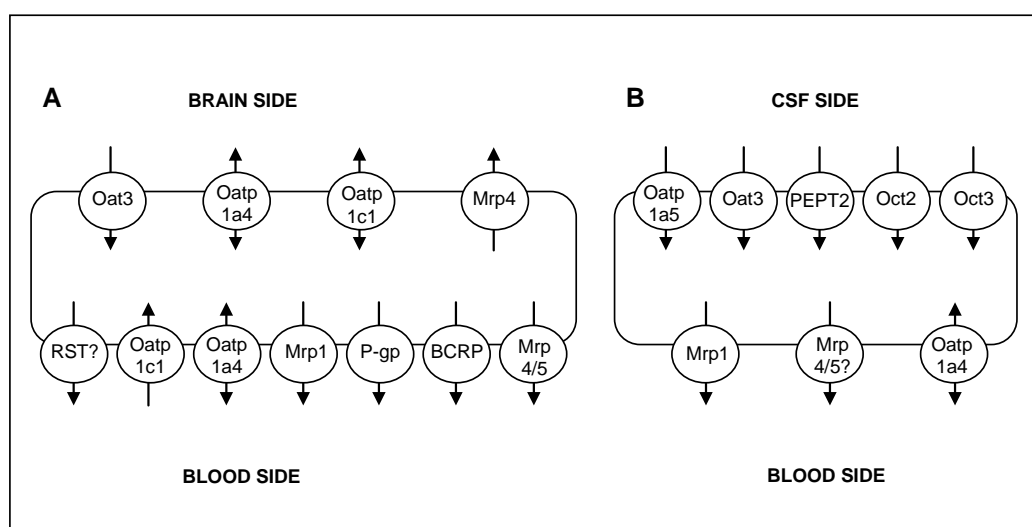
Figure 2.3. Simplified diagram of the efflux barrier at the BBB (A) and at the BCSFB (B). BCRP = breast cancer resistant protein, Mrp = multidrug resistant-associated protein, Oat = organic anion transporter, oatp = organic anion transporting polypeptide, oct = organic cation transporter, PEPT = peptide transporter, P-gp = P-glycoprotein, RST = renal specific transporter (Kusuhara and Sugiyama 2004, Kusuhara and Sugiyama 2005).

It has been traditionally believed that the role of the BCSFB in drug delivery to the CNS is not as significant as the role of the BBB. This belief is largely based on the smaller surface area of the BCSFB. However, this view has been challenged e.g. the vascular perfusion of the choroid plexus is approximately eight times higher compared to that of the total brain, and the choroid plexus also has larger surface area than thought previously - the estimated total apical surface area of the choroid plexus (75 cm^2 for 30-day-old rats) is very similar to the estimate for the surface area of the cerebral capillaries (155 cm^2 for 30-day-old rats) (Keep and Jones 1990, Faraci *et al*. 1994, Johanson *et al*. 2005). Furthermore, it is known that the vitamin C supply to the CNS occurs solely via the choroid plexus and not across the BBB (Rice 2000) emphasizing the importance of the BCSFB in the CNS delivery of some compounds. Therefore, recently it has been claimed that not only the BBB, but also the BCSFB seems to be exploitable for drug delivery to the brain (Johanson *et al*. 2005).

2.1.1.3 Circumventricular organs

In the CNS, the so-called circumventricular organs (CVOs) are a family of organs that lack the typical features of the BBB, such as the endothelial tight junctions. Therefore, their capillaries are generally highly permeable to blood-borne compounds (Rowland *et al*. 1991). The choroid plexus is one of the CVOs. The other CVOs are located on the walls of the 3rd and 4th ventricles and include the pineal gland, subfornical organ, subcommissural organ, organum vasculosum of the laminar terminalis, the area postrema, median eminence and neural lobe of the hypophysis. The CVOs possess neuroendocrine-type functions. They receive signals from and secrete products into both plasma and CSF. In other words, at the level of the CVOs, the brain can interact rapidly with blood-borne solutes (Begley 2004). Further diffusion of these blood-borne solutes within the brain is limited by the surrounding ependyma layer where adjacent cells form tight junctions (Rowland *et al*. 1991). In addition, metabolic enzymes are abundant in the CVOs, forming an enzymatic barrier between blood and the rest of the CNS (Ghersi-Egea *et al*. 1995).

2.1.2 Factors affecting drug permeation into the central nervous system

CNS permeation of drugs is an important issue in modern drug development because many new agents developed for CNS disorders cannot permeate into the CNS and therefore, cannot reach their site of action. The reason why the CNS permeation of drugs is so crucial is that the transvascular route remains the most desired way to deliver drugs to the CNS (Pardridge 2003). The other routes, i.e. intraventricular, intraparenchymal, intrathecal and nasal route may be advantageous occasionally but are unlikely to displace the transvascular route as the most common route of drug administration to reach the CNS (Huynh *et al*. 2006, Begley 2004).

Basically, there are four mechanisms that a compound may use in order to gain access to the CNS (Tsuji 2000), i.e. transcellular passive diffusion, carrier-mediated transport, receptor-mediated transcytosis and adsorptive-mediated transcytosis (Figure 2.2). Usually small molecular weight compounds enter via passive diffusion or carrier-mediated transport mechanisms whereas large molecules such as proteins utilize receptor- or adsorptive-mediated mechanisms. The ability of the drug to pass the BBB or the BCSFB by passive diffusion depends greatly on the physicochemical properties of the drug whereas the carrier- or receptor-mediated mechanisms require specific interactions between the drug and the transport protein or receptor.

2.1.2.1 Physicochemical properties of the drug

There is a well-established relationship between increasing lipid solubility and increasing BBB penetration with regard to passive transport (Levin 1980). The lipophilicity of a

molecule can be evaluated by determining its logarithmic octanol-water partition coefficient (log P_{oct}). Historically, based on biological activity data, a log P value of near 2 has been believed to be optimal for brain uptake. In a recent study with a set of CNS drugs with a wide range of calculated log P_{oct} values (from -0.2 to 6.1) a nonlinear relationship was observed between lipophilicity and *in situ* brain permeability values determined using an *in situ* brain perfusion method in rats (Summerfield *et al*. 2007). When calculated log P_{oct} was less than 2-3, a linear relationship was observed, but with calculated log P_{oct} values higher than approximately 3, the increase in the log P_{oct} no longer resulted in an increase in the *in situ* brain permeability values and a plateau effect was observed .

Ionization (i.e. charge) of a compound affects its ability to enter the CNS (Brodie *et al*. 1960). It is generally believed that charged molecules cannot penetrate through the BBB/BCSFB. This would mean that the CNS permeation of drugs that are partially ionized at the pH of the circulation will be determined by the unionized fraction of the drug. In addition, certain molecular characteristics such as polarity, polar surface area, Lewis bond strength, potential for hydrogen bond formation, molecular weight or volume, can influence the ability of a compound to cross the BBB/BCSFB (Begley 2004). These characteristics can be used to predict brain uptake of drugs and it has been suggested that descriptors such as hydrogen bond descriptors should be used instead of commonly used log P values in order to obtain better correlations with BBB/BCSFB permeability (Abraham and Platts 2000).

Generally, molecules that are both lipid soluble and have molecular weight less than a threshold of 400-600 Da can readily penetrate through the BBB/BCSFB, the extent of penetration being in proportion to their lipid solubility (Pardridge 1995). Molecules that do not exhibit favorable physicochemical properties may still penetrate into the CNS if they can take advantage of carrier-mediated transport mechanisms at the BBB/BCSFB (see chapter 2.1.2.3). Conversively, the CNS penetration of a compound may be far less than would be predicted based on its lipid solubility, for example if it is a substrate for the efflux mechanisms present at the BBB and the BCSFB, which have been discussed earlier in context of the efflux barrier (chapter 2.1.1.1).

2.1.2.2 Pharmacokinetics of the drug

Pharmacokinetics in the periphery affects the CNS permeation of drugs by affecting the amount of a drug available for uptake. Several factors such as peripheral distribution volume, extent of metabolism and plasma protein binding greatly affect the CNS availability of a drug after its systemic administration (Pardridge 1995).

Traditionally it has been believed that only the fraction of the drug that is not bound to plasma proteins is able to pass through biological membranes including the BBB and the BCSFB (the "free drug rule") (Rowland and Tozer 1995). Thus, the CNS delivery of drugs

that are partially bound to plasma proteins is greatly influenced by plasma protein binding. It is appreciated that both the equilibrium state of protein-drug binding and the rate of dissociation of a drug from the protein affect the drug transfer across the blood-CNS barriers (Fenstermacher 1989). This means that if the rate of dissociation is slow relative to the capillary transit time of plasma proteins, drugs that are bound to plasma proteins may not be available for transfer across the blood-CNS barriers whereas protein-bound drugs whose rate of dissociation is rapid may become free during their passage through the cerebral capillaries and consequently become available for transfer.

Some discrepancy exists regarding the "free drug rule" in the sense that it has been suggested that the unbound fraction may not explain the CNS uptake *in vivo* and also the protein-bound fraction may still be available for transport across the BBB to some extent (Tanaka and Mizojiri 1999, Jolliet *et al*. 1997). The proposed explanation to the exception for the free drug rule is that a conformational change occurring in albumin when it enters brain capillaries could lead to enhanced dissociation of the drug from protein (Tanaka and Mizojiri 1999, Pardridge 1995). These observations have been demonstrated using the brain uptake index and *in situ* brain perfusion methods. However, in a steady-state condition after intravenous administration (Dubey *et al*. 1989) or by using an *in situ* brain perfusion method with a modified Kety-Crone-Renkin (parallel tube) model (Mandula *et al*. 2006) no enhancement in the dissociation of the drug from protein was observed.

Ideally, at steady-state, the unbound drug concentration in the brain or CSF and blood should be identical. In other words, the clearances into and out of the brain or CSF are equivalent. However, this kind of equilibrium may never be reached if the efflux clearance (CL_{out}) exceeds the influx clearance (CL_{in}). Factors that influence the CL_{out} include active efflux mechanisms, metabolism in the CNS and the continuous CSF turnover (Hammarlund-Udenaes 2000).

2.1.2.3 Transport mechanisms at the blood-central nervous system interfaces

The BBB and the BCSFB express various influx transporter mechanisms. These transporters enable the CNS delivery of compounds which are fundamental to the normal functions of the brain but are restricted from CNS entry by passive diffusion due to their polar nature. Transport systems, which are shown to act as influx transporters at the BBB or at the BCSFB, include transporters for glucose, amino acids, monocarboxylic acids, organic cations, nucleosides, peptides and vitamin C (Tamai and Tsuji 2000). Some of the transporters at the blood-CNS barriers can be expressed in both sides of the barrier, and some of them act only as influx or as efflux transporters. Therefore, unambiguous classification to influx and efflux transporters is not appropriate, and some of the efflux transporters discussed in chapter 2.1.1.1 may act also as influx transporters.

Endogenous transport mechanisms can have relevance in the CNS delivery of drugs. A classical example of a drug which is delivered to the CNS via carrier-mediated transport is levodopa. In the body, including the brain tissue, levodopa is decarboxylated to the active compound dopamine but only levodopa is a substrate for the large neutral amino acid transporter (LAT1) and is thus able to reach the brain, the site of action (Tamai and Tsuji 2000). Many drugs have been suggested to enter into the CNS via carrier-mediated transport mechanisms, and undoubtedly, in the future more and more drugs will be shown to be transported across the BBB or BCSFB via some specific transport protein (Dobson and Kell 2008). In addition to levodopa, examples of drugs that are delivered into the CNS via carrier-mediated transport include the anticancer agent melphalan and particularly its lipophilic analogue DL-NAM via LAT1 (Takada *et al.* 1992), the anticonvulsant agent, valproic acid via the transporter for medium-chain fatty acids (Adkison and Shen 1996), active forms of HMG-CoA reductase inhibitors simvastatin and lovastatin via the transporter for monocarboxylic acids (Tsuji *et al*. 1993), and the H1-antagonist mepyramine via the transporter for cations (Yamazaki *et al*. 1994).

The BBB transport of large molecules, such as proteins, is dependent on specific receptors present at the BBB. For instance, the CNS delivery of iron and insulin occurs via receptor-mediated transport (RMT) by transferrin and insulin receptors (Tsuji 2000). These receptors may be useful when delivering large molecular-weight therapeutic agents such as proteins or genes into the CNS (Pardridge 2002). Another transport mechanism for large molecules across the BBB is the absorptive-mediated transcytosis (AMT) which is less specific than the receptor-mediated transcytosis. AMT is based on electrochemical interactions between negatively the charged cell membrane and a positively charged moiety of the molecule (Tsuji 2000). While there are reasonably promising data available about the brain delivery of large molecular weight compounds using the RMT mechanism in animal models, the clinical significance and the usefulness of RMT (and AMT) to target CNS delivery is yet to be seen as there are several issues to be resolved before these techiques can be successfully and safely transferred to humans (de Boer and Gaillard 2007).

2.1.2.4 General view about drug delivery into the central nervous system

One of the factors affecting the CNS permeation of compounds is the cerebral blood flow. The cerebral blood flow and the capillary surface area may differ in different brain regions, and therefore, it can be presumed that this could lead to regional differences in brain uptake and disposition of drugs. A change in the blood flow is likely to be particularly relevant for those drugs whose brain uptake is fast and limited by the blood flow. The capillary surface area i.e. the number of perfused capillaries may, at least in theory, affect the CNS permeation of both highly and poorly permeable drugs (de Lange and Danhof 2002).

Individual differences in humans may also have an impact on the CNS delivery of drugs. For instance, several diseases, such as stroke, brain tumours, infectious and inflammatory processes, Alzheimer's disease, Parkinson's disease, multiple sclerosis, HIV, and epilepsy may disrupt the intactness of the BBB (Abbott *et al*. 2006). Even peripheral inflammation stimuli have been shown to produce an increase in the BBB permeability (Huber *et al*. 2001). However, the consequences of this disease-related BBB disruption in CNS delivery of drugs remains to be clarified (de Lange and Danhof 2002). In addition, it can be hypothesized that individual variability in the expression of transport proteins at the blood-CNS barriers could have a role in the CNS delivery of drugs which are substrates for transport proteins. Figure 2.4 shows some factors affecting the CNS transport of drugs.
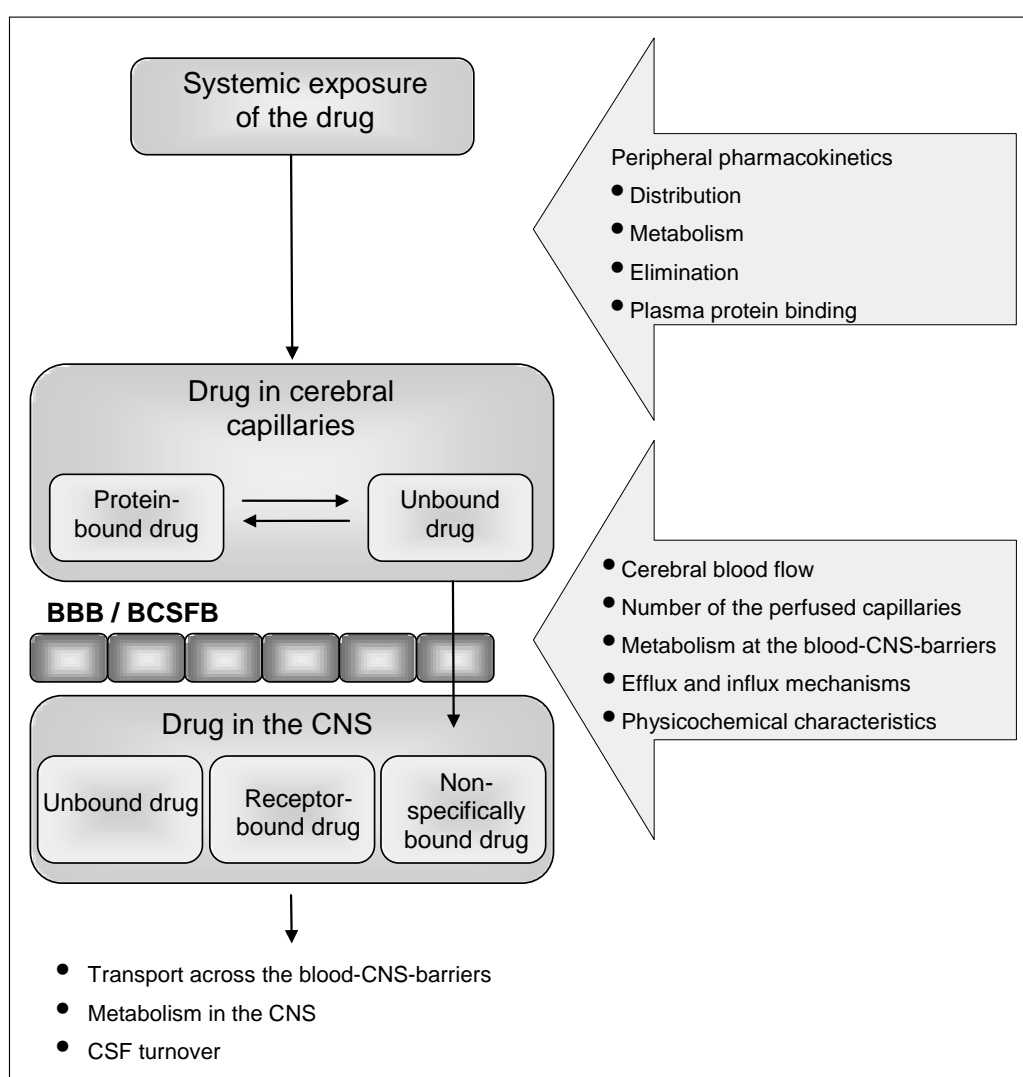
Figure 2.4. Factors affecting the rate and extent of CNS transport of drugs.

It has to be remembered that from the CNS drug delivery view in general, both the *rate* of transport across the blood-CNS barriers and the *extent* of the CNS distribution are of importance. Furthermore, they should be understood as two separate processes which may be governed by different factors (Hammarlund-Udenaes 2000). For instance, a slow rate of transport across the blood-CNS barriers does not necessarily lead to a low level of drug in the CNS, and vice versa. The *rate* of transport is influenced largely by the physicochemical characteristics, and further, the permeation properties of the drug. For highly permeable drugs, the cerebral blood flow may become the rate-limiting factor. The influx rate of drugs is also affected by the efflux mechanisms at the BBB or BCSFB. In a recent study by Liu *et al.* (2005), it was suggested that also brain tissue binding has a role in the rate of CNS entry. When the rate was evaluated by the time to reach brain equilibrium, rapid CNS entry requires both high BBB permeability and low brain tissue binding. The *extent* of CNS distribution is determined by the CL_{in} and CL_{out} of a drug (Hammarlund-Udenaes 2000). Factors that influence the ratio between CL_{in} and CL_{out} include active influx or efflux mechanisms, metabolism in the CNS and the continuous CSF turnover. In addition, among lipophilic compounds, brain tissue binding has an effect on the uptake across the BBB as brain tissue binding may help to maintain a diffusion gradient across the BBB (Summerfield *et al.* 2007). As for total brain drug concentration as well as total brain to total plasma concentration ratio, drug binding in both plasma and in brain tissue has a crucial role (Liu *et al.* 2005, Summerfield *et al.* 2006).

Finally, as discussed in chapter 2.1.1., the CNS consists of various compartments and there are several pathways for compound transfer between the different compartments. The drug concentration can vary in different parts of the CNS and for instance nonspecific binding in the brain parenchyma as well as binding to specific receptors can affect the drug concentration in the CNS. Once present in the CNS, the drug is distributed into the extracellular and intracellular compartments. Extracellular brain concentrations are dependent on the intracellular distribution of a drug and it is drug-specific whether the extracellular or the intracellular drug concentration is more relevant (de Lange and Danhof 2002).

2.1.3 Methods for improving central nervous system drug delivery

Many methods have been described in the literature for improving the CNS delivery of drugs that lack satisfactory BBB or BCSFB penetration properties. These methods include direct administration of a drug to its site of action through intracerebral or spinal injection or infusion, delivery via the olfactory route, transient modulation of the BBB with hypertonic solutions, with vasoactive agents or with ultrasound and electromagnetic radiation, cell-penetrating peptide vectors and liposomes and nanoparticles (Begley 2004). All of the existing methods have their own benefits and drawbacks. Many of them have already been used successfully to improve the CNS delivery of a drug, as is the case in treating human

brain tumours. In the present literature review, two methods for improving the CNS of therapeutics are described in more detail – lipidization of the drug as one of the traditional, chemical based approaches, and influencing the endogenous transport systems at the blood-CNS barriers since this technique has been partially applied in the experimental studies.

2.1.3.1 Lipidization of the drug

Optimization of the physicochemical properties of the drug molecule is a convenient way to improve its CNS delivery, since the blood-CNS barriers allow compounds to enter the CNS via passive diffusion only through the transcellular route. Enhancement of the CNS delivery has been achieved in several ways, e.g. by designing a lipid soluble analog of the drug, by using brain-targeting chemical delivery systems (Bodor and Buchwald 2002) or by increasing temporarily the lipophilicity of the drug molecule by the prodrug approach (Anderson 1996, Greig 1989). For instance, as a strategy to improve the CNS delivery of NSAIDs, a triglyceride prodrug of ketoprofen has been designed and evaluated in various animal models (Deguchi *et al*. 2000). It was discovered that following the administration of the triglyceride prodrug the $AUC_{(brain)}$ value of ketoprofen was increased when compared to administration of ketoprofen itself, and also that the brain uptake of the prodrug across the BBB was higher than that of ketoprofen. These results suggested that the brain delivery of ketoprofen could be improved via this prodrug approach. The prodrug approach has been used also in attempts to improve the CNS delivery of an anticancer agent chlorambucil (Greig *et al*. 1990). A series of chlorambucil esters was prepared and the chlorambucil-tertiary butyl ester displayed an increased brain delivery compared to chlorambucil, and as a result, the brain to plasma concentration integral ratio of total active compounds derived from the prodrug was 35-fold greater than that derived from chlorambucil administration. However, of the total active compounds monitored in the brain, the chlorambucil-tertiary butyl ester itself (which has some alkylating activity) comprised the majority. This indicates that the ester prodrug exhibited slow cleavage rate in the brain tissue, and illustrates the challenges related to the prodrug approach – in order to enhance the CNS delivery, the prodrug needs to have optimal bioconversion rates both in the systemic circulation and in the CNS. Too rapid bioconversion rate in the systemic circulation may result in complete conversion of the prodrug to the parent compound before sufficient prodrug reaches the CNS, whereas too slow bioconversion rate in the CNS may mean that the parent compound is released too slowly compared to the rate at which the parent compound (or the prodrug) is cleared fron the CNS by passive diffusion back to the systemic circulation, CSF bulk flow or other clearance mechanisms (Anderson 1996).

Unfortunately, increase in the lipophilicity of a drug - either by designing a more lipophilic analog or by a prodrug approach - may lead not only to improved BBB permeation but also to altered peripheral pharmacokinetics, e.g. improved permeation to peripheral organs, increased plasma protein binding and elimination. These undesirable effects on peripheral pharmacokinetics can decrease the plasma concentrations of the drug, and ultimately, this

may offset the positive effect of increased BBB permeability and the overall CNS availability may not be increased (Pardridge 2003). In addition, an increase in the lipophilicity can lead to an increase in the affinity for the efflux transporters at the BBB (Leisen *et al*. 2003) as well as to an increase in the non-specific binding in the brain tissue, all of which can lead to decreased efficacy (Summerfield *et al*. 2006, Summerfield *et al*. 2007).

2.1.3.2 Influencing the endogenous transport systems

Endogenous transport mechanisms can have relevance in the CNS delivery of drugs because they can be utilized in the CNS uptake of drugs. On the other hand, inhibition of efflux proteins may enhance the CNS uptake of drugs whose permeation into the CNS is limited by efflux mechanisms.

One way to utilize the endogenous influx transporters at the blood-CNS barriers is to design a drug which closely resembles the endogenous substrate of the transport protein and is thus recognized and transported into the CNS. A classical example of a drug which is delivered to the CNS via carrier-mediated transport is the LAT1 substrate, levodopa (Tamai and Tsuji 2000). The prodrug approach can also be applied in order to utilize carrier-mediated transport across the blood-CNS barriers. There are some prodrugs designed to target various membrane transporters at blood-CNS barriers. For instance, there is an amino acid prodrug of a glycine-NMDA receptor antagonist 7-chlorokynurenic acid which has been shown to be transported across the BBB by LAT1 (Hokari *et al*. 1996). Recently, it was reported that the amino acid prodrug of ketoprofen could also be transported across the BBB via LAT1, evidence that conjugation of a drug and a LAT1-substrate could promote carrier-mediated drug delivery to the brain (Gynther *et al*. 2008). In addition, in a prodrug approach where 7-chlorokynurenic acid was conjugated with D-glucose, it was suggested that the prodrug could be transported across the BBB via the glucose carrier protein (Bonina *et al*. 2000, Battaglia *et al.* 2000). Also in the study by Polt *et al*. (1994) it was hypothesized that the attachment of β-D-glucose moiety to an enkephalin analogue would be one way to increase the transfer of the peptide across the BBB since after peripheral administration of the compound, significant analgesia was achieved. In addition to LAT1 and glucose transporters, the ascorbate transporter has been studied as a possible route through which to deliver drugs into the CNS (Manfredini *et al*. 2002).

The concept of efflux inhibition has been mainly used to enhance the CNS delivery of anti-tumour or anti-HIV agents. Fellner *et al*. (2002) have demonstrated using nude mice that the brain entry and the therapeutic effect of paclitaxel could be enhanced by simultaneous administration of a P-gp inhibitor valspodar. In addition, another P-gp inhibitor, GF120918 was demonstrated to increase the CNS entry of drugs including nelfinavir, morphine and amprenavir in rats or mice (Polli *et al*. 1999, Savolainen *et al*. 2002, Letrent *et al*. 1999).

Probenecid which is a non-specific inhibitor of various transporters at the blood-CNS barriers has been shown to increase the brain concentrations of dideoxynucleosides in rats (Galinsky *et al*. 1991). In patients with bacterial meningitis, co-administration of probenecid increased the CSF levels of amoxicillin and may have had an effect on elimination of amoxicillin from the CSF (Craft *et al*. 1979).

Research in the field of efflux inhibitors has suffered from the lack of specific inhibitors. For instance, the commonly used efflux inhibitor probenecid is extremely non-specific which complicates the interpretation of the results from preclinical studies. Other problems related to the clinical use of efflux inhibitors include the possibility that the inhibitor may alter the pharmacokinetic properties of the drug, leading to potential toxicity. It is also possible that high doses of inhibitors may be required in order to obtain efflux inhibition; often very high concentrations have been used in pre-clinical studies to illustrate the effects of efflux inhibitors. The requirement of a high dose may impede the clinical use of efflux inhibitors. In addition, the ability of efflux inhibition to enhance the CNS delivery of an efflux protein substrate may be rather limited (Kalvass and Pollack 2007).

2.1.4 Experimental models for studying central nervous system drug delivery

In vitro BBB models include isolated brain capillaries, primary or low passage brain capillary endothelial cell cultures, immortalized brain endothelial cells and immortalized artificial membranes (Gumbleton and Audus 2001, Nicolazzo *et al*. 2006). Of the endothelial cell cultures, cells from bovine and porcine sources are the most commonly used. Cells of non-cerebral origin have also been used but they may suffer from serious limitations. For instance, it has been suggested that the human colon carcinoma cell line (Caco-2) predicts poorly the *in vivo* BBB permeability (Lundquist *et al*. 2002). The basic principle of an *in vitro* BBB model suitable for studying general mechanisms of transport through endothelial cells is presented in Figure 2.5.

Obviously, the greatest challenge is to develope a model which faithfully and accurately replicates the *in vivo* conditions, e.g. the same kind of integrity as the BBB and same amount and composition of transport proteins and enzymes. Pardridge *et al*. have studied the correlation between drug transport across primary cultures of bovine brain capillary endothelial monolayers and BBB permeability after brain perfusion. According to their results, there is a strong correlation between permeability values measured in endothelial monolayers and after brain perfusion. However, the permeability values in the *in vitro* system were on average about 150-fold greater than those determined after brain perfusion (Pardridge *et al*. 1990). This correlation did not apply to drugs that pass across the BBB via carrier mediated transport indicating that there had been a loss of activity of transport proteins in the *in vitro* system.

In order to improve the tightness of the *in vitro* BBB models, astrocyte-conditioned medium or co-culture with astrocytes has been used (Gumbleton and Audus 2001). The so-called conditionally immortalized cells may also represent a useful tool in CNS drug delivery research, especially when transport systems are concerned (Terasaki *et al*. 2003).
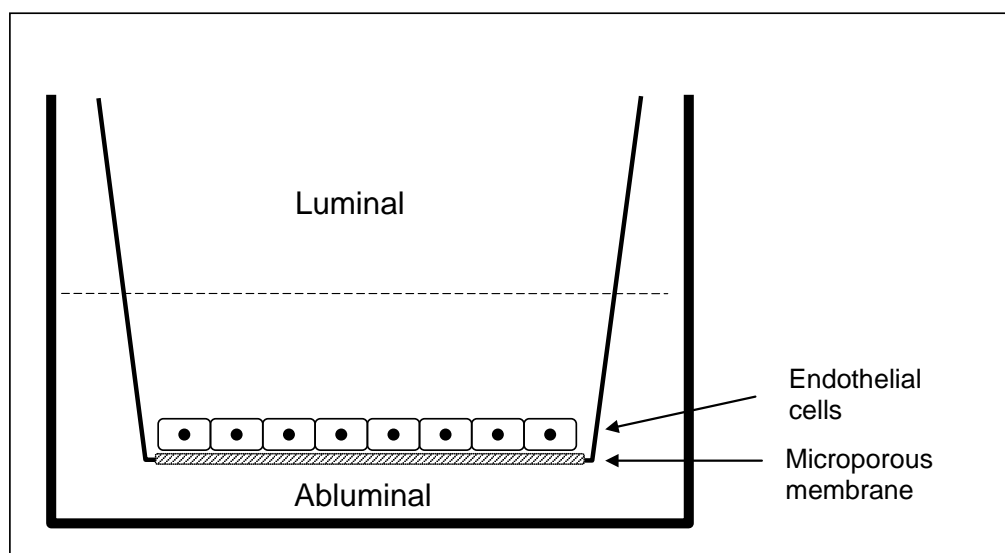
Figure 2.5. Diagram of the basic principle of an *in vitro* BBB model. Endothelial cells are grown on microporous membranes. Astrocytes can be grown on the underside of the same membrane or on the bottom of the chamber to release soluble factors, which preserve some of the BBB properties. The test compounds can be added to the luminal or to the abluminal compartment, and bidirectional transport across the BBB can be studied.

In vivo **and** *in situ* the BBB permeability studies can be basically carried out through two different approaches; the single pass uptake approach and the continuous uptake approach (Smith 1989). In the single pass uptake approach, the brain uptake or extraction of a drug is determined after a bolus injection into the carotid artery followed by its single pass through cerebral capillaries. Techniques most often used to evaluate the influx of drugs into the brain using the single pass uptake approach include the indicator diffusion technique, the brain uptake index (BUI) technique and the single injection-external registration technique. Common to all these techniques is their relative simplicity but one major disadvantage of these methods is their limited sensitivity due to the short uptake time (5-15 seconds). A scheme of the most representative single pass uptake method, BUI, in presented in Figure 2.6. The continuous uptake approach overcomes the sensitivity-problems of the single pass uptake approach since the uptake can be measured over a prolonged period of time. The two basic techniques used to evaluate the influx of drugs into the brain using the continuous uptake approach are the intravenous administration technique and the brain perfusion technique.

Since these techniques have been utilized in the present study they are discussed in more detail in the following chapters.
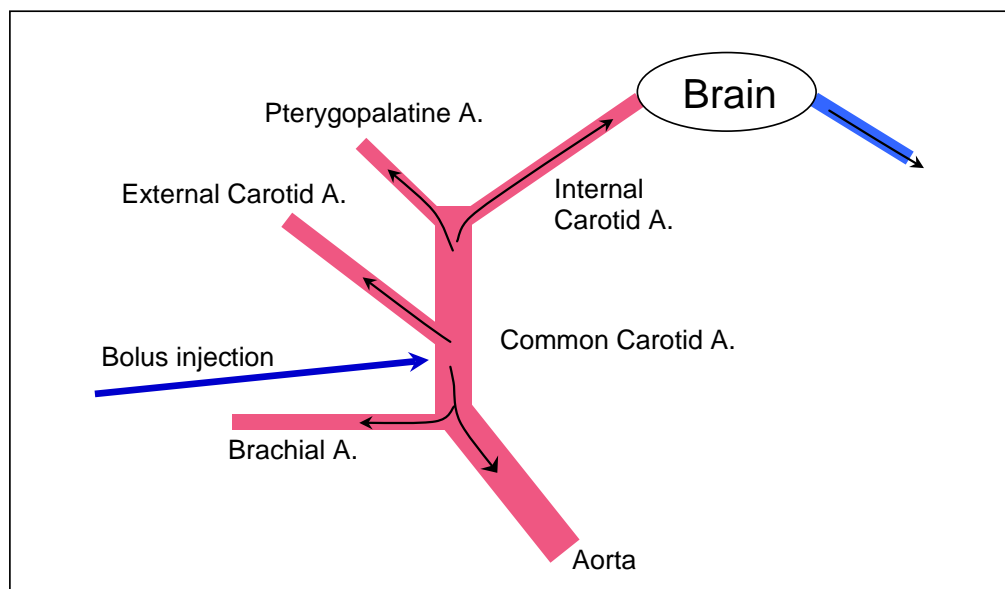
Figure 2.6. In the BUI method, the drug is injected as a bolus dose into the common carotid artery together with a freely permeable internal standard. The arrows represent the diffusion of the test mixture in the plasma compartment. The internal standard is used to define the amount of injected mixture which reaches the brain. At 5-15 seconds post-dose, the brain is removed and the brain uptake of the drug is determined by the ratio of the internal standard to drug in the brain to the ratio of the internal standard to drug in the dosing mixture. (Bonate 1995)

Some of the techniques used to estimate the brain uptake of drugs can also be used to studying the efflux of drug from brain to blood. For instance, the modified version of the brain uptake index technique (Bonate 1995) and *in situ* brain perfusion technique (Chikhale *et al*. 1995) have been utilized in efflux studies. The brain efflux index technique, where the test compound and an impermeable reference compound are microinjected directly into the brain and their brain concentrations are measured over time, has also been used for studying efflux mechanisms at the BBB (Kakee *et al*. 1997).

2.1.4.1 *In situ* brain perfusion technique

The *in situ* brain perfusion method was originally described by Takasato *et al*. in 1984 (Takasato *et al*. 1984). In this method, the right cerebral hemisphere of a rat is perfused by retrograde infusion of perfusion fluid into the right external carotid artery (Figure 2.7). The

perfusion rate is adjusted so that the effect of systemic blood flow to the right hemisphere is minimized and that the carotid perfusion pressure is maintained below the 160-190 mmHg threshold that damages the BBB. At predetermined times, brain tissue is collected and the amount of the compound is determined. Finally, the brain uptake kinetics can be analyzed and transport or permeability constants can be calculated.

Subsequently the Takasato method was modified by several research groups. For instance, in the modification described by Smith and Allen (2003) the method became surgically less time-consuming as the perfusion fluid catheter was inserted into the common carotid artery, followed by ligation of the external carotid artery (Figure 2.7). The pterygopalatine artery could either be ligated or left open. In addition, in the modified method, blood flow from the systemic circulation is stopped by severing cardiac ventricles at the beginning of the perfusion. This modification makes the method less vulnerable to the perfusion flow rate as there is no contribution to flow from the systemic circulation. Prior to perfusion, the blood supply to the brain is maintained via the crossover of blood from the contralateral carotid artery at the Circle of Willis (Figure 2.7).

The modification introduced by Triguero *et al*. (1990) incorporated the possibility to differentiate between a transcytosed compound from simple binding and/or endocytosis of the compound by brain capillary endothelium. The method referred to as the capillary depletion method used a dextran density centrifugation step to deplete brain homogenate of the vasculature.

The most crucial advantage of the *in situ* brain perfusion method is the ability to control the composition of the perfusion fluid. This allows the investigation of carrier-mediated transport mechanisms at the BBB as well as the effect of plasma protein binding. The perfusion fluid can be manipulated in many ways such as through changes in the solute concentrations, pH, osmolality, ionic contents and protein compositions. In addition, there is little (< 5%) or no contribution of systemic blood flow to the perfused hemisphere and the contribution of peripheral pharmacokinetics is avoided (Takasato *et al*. 1984).

The disadvantages related to the *in situ* brain perfusion method include the impossibility to control the drug concentration in brain extracellular fluid, the need for an appropriate analytical assay for the determination of drug concentration in brain tissue, possible disruption in the physiological function of the BBB during the experiment and the use of analgesics during the experiment which may modify the results (Smith 1996).
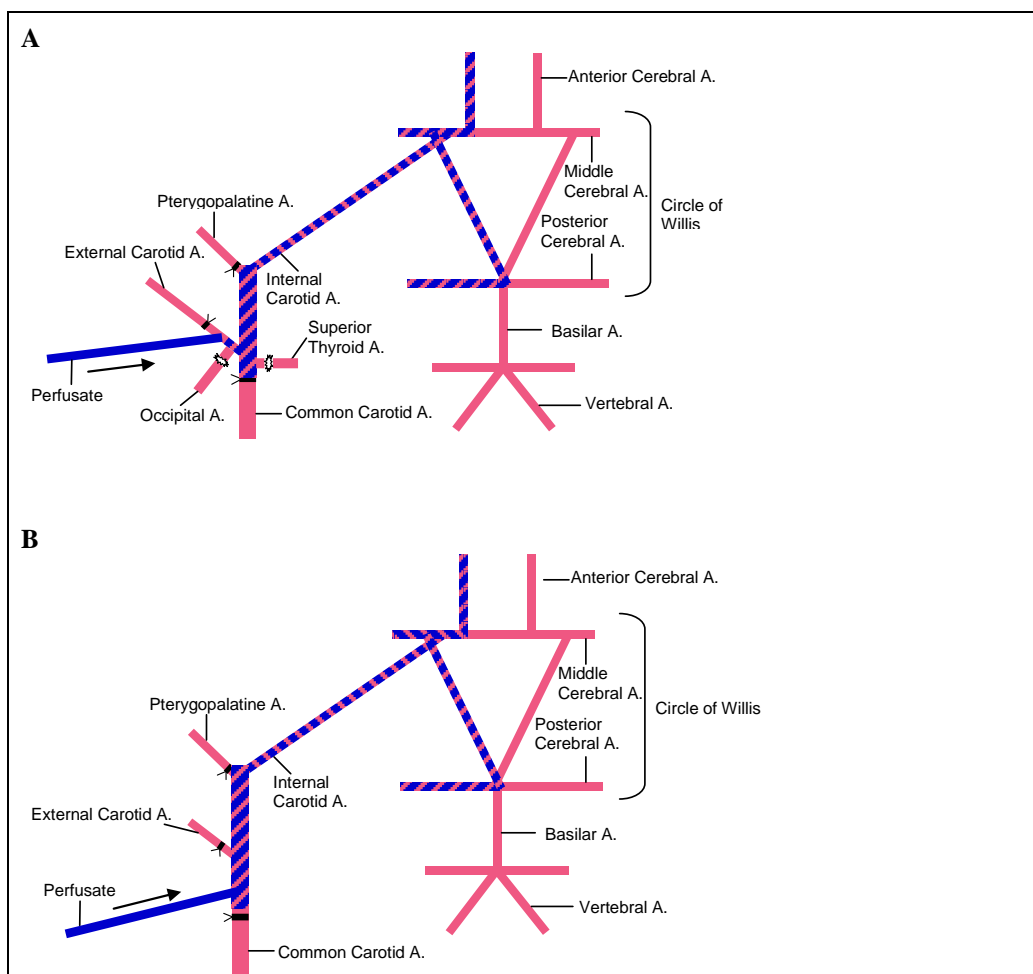
Figure 2.7. A. In original Takasato method (1984), the occipital and superior thyroid arteries are coagulated and cut, and the right pterygopalatine artery is ligated. The external carotid artery is catheterized and the common carotid artery is ligated immediately prior to the perfusion. B. In the modified method by Smith and Allen (2003) the common carotid artery is catheterized and the external carotid artery is ligated. The pterygopalatine artery is ligated or it can be left undisturbed.

2.1.4.2 Intravenous administration technique

In the intravenous administration technique, the drug is injected intravenously as a bolus injection or as a constant infusion, and the amount of the drug in brain is measured after a predetermined time. Simultaneously with the brain sampling, a blood sample is collected in order to detect the whole blood or plasma concentration of the drug. The uptake time can range from about 10 seconds to as long as several hours, depending on the brain permeability

properties of the drug (Smith 1989). The amount of the drug in brain tissue can be determined after tissue sampling followed by a suitable analytical technique or by different imaging techniques, and finally, the BBB permeability can be calculated using kinetic models. In addition to BBB pharmacokinetics, also the systemic pharmacokinetics can be obtained, this being regarded as one of the main advantages of this technique (Bickel 2005). The possibility to infuse drugs to steady-state further increases the value of the intravenous administration technique. In addition, the intravenous administration technique allows the evaluation of the CSF permeation of drugs since in addition to the blood and brain tissue sampling, the CSF can be collected at predetermined times.

Intravenous administration technique models most closely mimic the *in vivo* situation as the BBB is intact and the functions of various transporters, enzymes etc. both at the BBB as well as in the periphery are not compromised (Bickel 2005). On the other hand, the presence of metabolism and peripheral pharmacokinetics may complicate the application of the technique and the interpretation of the results obtained using the technique. For instance, differentiating between unidirectional and bidirectional transport can be a great challenge (Bickel 2005).

2.1.4.3 Other experimental models for studying central nervous system drug delivery

One of the most recent and important additions to the battery of *in vivo* methods available to study CNS delivery is the intracerebral microdialysis technique. This technique involves the stereotactic implantation of a semipermeable microdialysis probe into the brain, and the drug concentration in the ECF compartment of the brain is determined by monitoring the drug concentration in the perfusate that flushes the microdialysis probe (Figure 2.8). With the intracerebral microdialysis technique, studying both influx and efflux mechanisms of drugs in freely moving animals is possible, and concentrations in brain ECF, CSF or blood can be measured simultaneously and furthermore, they represent directly the unbound concentration of the drug (Hammarlund-Udenaes 2000). Other advantages of this method include the possibility to obtain a concentration-time course of the test compound from individual subjects, and the possibility to determine local drug concentration in the CNS, as the probe can be placed in any region of the brain (de Lange et al. 1999). The limitations with the intracerebral microdialysis method include the potential disruption of the BBB resulting from the probe implantation, the requirements for the assay method (adequate sensitivity) and the need for assessing the relationship between dialysate concentrations and the brain ECF concentrations (the so called *in vivo* concentration recovery) (de Lange *et al*. 1999).
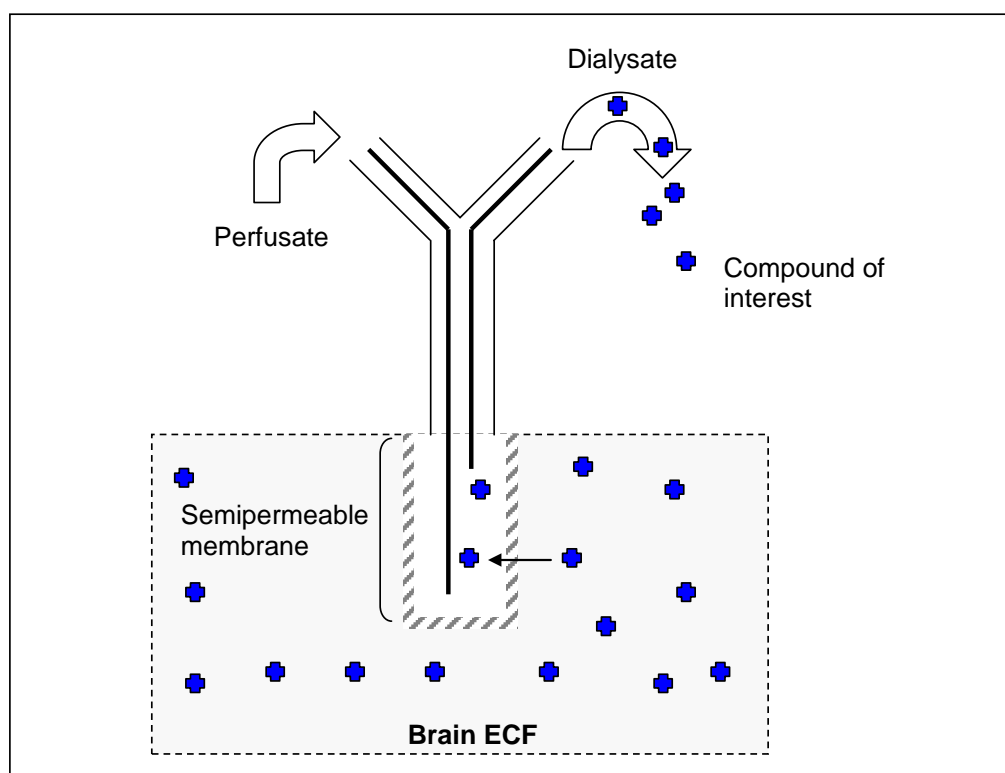
Figure 2.8. Schematic drawing of the principle of microdialysis. The microdialysis probe is implanted into brain and the semipermeable membrane is perfused with a physiological solution at a constant flow rate. The diffusion of compounds occurs according to the concentration gradient.

In addition, imaging techniques such as quantitative autoradiography, magnetic resonance imaging (MRI), positron emission tomography (PET) and single photon computed tomography (SPECT) expand greatly the selection of methods which can be used in the area of CNS research because the noninvasive techniques MRI, PET and SPECT can also be applied in humans (Bickel 2005). Finally, *in silico* models which could accurately predict the CNS permeation of drugs are eagerly awaited. In particular, the modeling of the CNS permeation of drugs which are substrates for influx and efflux transporters at the blood-CNS barriers is currently impossible. The development of reliable models with good abilities to predict the CNS permeation of all kinds of drugs requires most of all the collation of more experimental brain permeation data to make the modeling more comprehensive (Clark 2003).

In summary, owing to the complexity of the CNS and the multiplicity of the factors that influence the CNS uptake of drugs, it is an enormous challenge to develop an ideal experimental model for studying the CNS drug delivery. Typically, compromises have to be made in order to model faithfully the *in vivo* situation and yet to retain adequate simplicity

and practicality. Often, the combined use of *in vivo*, *in situ* and *in vitro* models is of great value (Johanson *et al.* 2005).

2.2 Non-steroidal anti-inflammatory drugs

2.2.1 Physicochemical and pharmacokinetic properties of ibuprofen, ketoprofen and indomethacin

NSAIDs are a chemically heterogeneous group of compounds which share common pharmacological effects. Ibuprofen and ketoprofen belong to the group of propionic acid derivatives whereas indomethacin is an acetic acid derivative (Figure 2.9). Some physicochemical properties of ibuprofen, ketoprofen and indomethacin are shown in Table 2.2.
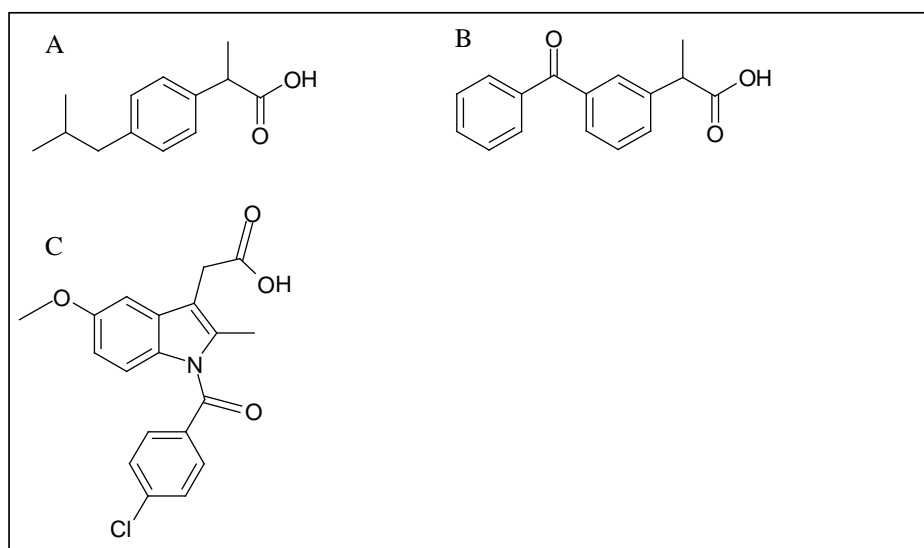
Figure 2.9. Structures of ibuprofen (A), ketoprofen (B) and indomethacin (C).

Table 2.2. Molecular weight, pKa and logP values of ibuprofen, ketoprofen and indomethacin.

Compound	Molecular weight	pKa[a]	logP[a]	Apparent logP (pH 7.4)	
				Calculated[b]	Experimentally determined
Ibuprofen	206	4.41	3.72	0.73	0.98 (Austin *et al*. 2002)
					1.07 (Barbato *et al*. 1997)
Ketoprofen	254	4.23	2.81	-0.36	-0.09 (Austin *et al*. 2002)
					-0.25 (Barbato *et al*. 1997)
					-0.2 (Rautio *et al*. 1998)
Indomethacin	358	4.17	3.11	-0.12	0.89 (Austin *et al*. 2002)
					0.91 (Barbato *et al*. 1997)

a. Advanced Chemistry Development (ADC/Labs) Software Solaris V4.67

b. Calculated according to equation $\log P = \log P_{7.4} - \log\left[\dfrac{1}{1+10^{pH-pKa}}\right]$

Most NSAIDs, including ibuprofen, ketoprofen and indomethacin, are rapidly absorbed from the gastrointestinal tract after oral administration. Peak plasma concentrations of ketoprofen and indomethacin are reached within 1-2 hours (Burke *et al*. 2006, Hucker *et al*. 1966, Ishizaki *et al*. 1980, Upton *et al*. 1981). Ibuprofen has been shown to reach a peak plasma concentration as quickly as 15-30 minutes after oral administration (Lee *et al*. 1985). In addition, absorption of these three NSAIDs is virtually complete.

In the circulation, ibuprofen, ketoprofen and indomethacin are extensively bound to plasma albumin. The unbound fraction of ibuprofen, ketoprofen and indomethacin is less than 1% in humans determined *in vitro* by equilibrium dialysis at 37°C (Netter *et al*. 1985, Bannwarth *et al*. 1990, Bannwarth *et al*. 1995). It has been suggested that ibuprofen and ketoprofen bind with high affinity to site II in human serum albumin whereas indomethacin binds to site I (Kragh-Hansen *et al*. 2002). Ibuprofen, ketoprofen and indomethacin have volumes of distribution of about 0.2, 0.1 and 0.3 l/kg, respectively (Burke *et al*. 2006, Upton *et al*. 1981). The estimated plasma half-life of indomethacin in humans after oral administration is about 2 hours (Hucker *et al*. 1966). However, presumably due to the enterohepatic cycling of indomethacin and its metabolites, a terminal half-life as long as 120 h has been described. However, this long half-life may be of no clinical relevance (Buxton 2006). The estimated plasma half-life of ketoprofen in humans is about 1.5 hours (Ishizaki *et al*. 1980, Upton *et al*. 1981) and that of ibuprofen about 2 hours (Lee *et al*. 1985). In rats the apparent half-lives of indomethacin and ketoprofen has been suggested to be about 4 and 10 hours, respectively, i.e. longer than in humans (Hucker *et al*. 1966, Foster and Jamali 1988, Satterwhite and Boudinot

1992), but the apparent half-life of ibuprofen has been suggested to be comparable in rats and humans (Satterwhite and Boudinot 1991).

Hepatic biotransformation and renal excretion are the main elimination pathways of the majority of NSAIDs in humans (Verbeeck 1990). Metabolism of indomethacin includes *O*-demethylation (50%), conjugation with glucuronic acid (10%), and *N*-deacylation (Burke *et al*. 2006). Indomethacin is excreted in the bile, reabsorbed and finally excreted in the urine (Hucker *et al*. 1966). The metabolism of ketoprofen involves the formation of acyl glucuronides, which are excreted in the urine (Ishizaki *et al*. 1980). Acyl glucuronides are unstable in physiological conditions and since they can be hydrolyzed back to the parent compound, accumulation of the acyl glucuronide metabolite of ketoprofen in the plasma can significantly delay the elimination of ketoprofen (Verbeeck 1990). Ibuprofen is mainly metabolized to hydroxylate or carboxylate derivatives (Burke *et al*. 2006). The renal excretion of unmetabolized ibuprofen, ketoprofen and indomethacin is a minor elimination pathway. Less than 1% of ibuprofen, 10% of ketoprofen and between 5 to 20% of indomethacin are excreted in an unchanged form (Burke *et al*. 2006, Hucker *et al*. 1966, Ishizaki *et al*. 1980).

2.2.1.1 Central nervous system permeation

Preclinical studies evaluating the CNS permeation of NSAIDs vary in the species used in the study, administration route, dose, and methods for measuring and evaluating the CNS uptake. In general, the *in vivo* CNS permeation of NSAIDs is believed to be quite limited. Table 2.3 shows some experimental data concerning the CNS permeation of NSAIDs in rodents.

Table 2.3. Experimental data on CNS permeation of NSAIDs in rodents.

NSAID	Study protocol	Outcome	Ref.
Ketoprofen	I.p. administration (5 mg/kg) in rats, blood and CSF samples collected 0.5-24 h after administration.	AUC_{CSF} / $AUC_{plasma, total}$ ratio: 0.006	(Matoga *et al*. 2002)
Naproxen Flurbiprofen	I.v. bolus (6 mg/kg) in rats, blood and brain tissue samples collected 10 and 60 min after administration.	Brain / plasma ratio (10; 60 min)[a]: Naproxen: 0.014; 0.017 Flurbiprofen: 0.005; 0.004	(Frijlink *et al*. 1991)
Ketoprofen	I.v. administration (40 µmol/kg) in mice, blood and brain tissue samples collected 1-60 min after administration.	AUC_{CSF} / AUC_{plasma} ratio: 0.017[a]	(Deguchi *et al*. 2000)
	In situ brain perfusion (10-60 s) in mice with buffer containing 30 µM ketoprofen and BSA.	CL_{in}: 0.0308 ml/min/g	

Table 2.3. continued…

NSAID	Study protocol	Outcome	Ref.
Ketoprofen Carprofen Fenoprofen Flurbiprofen Ibuprofen Naproxen Suprofen Tiaprofenic acid	I.p. administration (5 mg/kg) in rats, blood and CSF samples collected 0.5-6 h after administration.	AUC_{CSF} / $AUC_{protein-free\ plasma}$ ratio: Ketoprofen: 0.64 Carprofen: 0.23 Fenoprofen: 4.79 Flurbiprofen: 5.31 Ibuprofen: 1.44 Naproxen: 0.96 Suprofen: 0.81 Tiaprofenic acid: 0.79	(Pehourcq *et al.* 2004)
Ibuprofen Flurbiprofen Indomethacin	*In situ* brain perfusion (10-30 s) in rats with buffer containing 0.54, 0.15; and 2.5 μM of ibuprofen, flurbiprofen and indomethacin, respectively and 0 or 2.7% BSA.	BBB PS_u-value from buffer: Ibuprofen: 2.63×10^{-2} ml/s/g Flurbiprofen: 1.60×10^{-2} ml/s/g Indomethacin: 0.64×10^{-2} ml/s/g 2.7% BSA decreased the brain uptake 95%	(Parepally *et al.* 2006)
Naproxen Ketoprofen Diclofenac Piroxicam Fenoprofen Sulindac Ibuprofen Flurbiprofen Meclofenamic acid	P.o. (50 mg/kg/day) for 3 days in Tg2576 transgenic mice.	Brain / plasma concentration ratio[a]: Naproxen: 0.02 Ketoprofen: 0.02 Diclofenac: 0.08 Piroxicam: 0.18 Fenoprofen: 0.02 Sulindac: 0.03 Ibuprofen: 0.05 Flurbiprofen: 0.03 Meclofenamic acid: 0.03	(Eriksen *et al.* 2003)
Celecoxib	P.o. (50 or 100 mg/kg/day) for 3 days in mice.	Brain / plasma concentration ratio: 3.0 and 2.4 for the dose of 50 and 100 mg/kg/day, respectively.	(Kukar *et al.* 2005)
Ibuprofen *R*-flurbiprofen Indomethacin Naproxen Sulindac sulfide Mefanamic acid Fenoprofen Methylflurbiprofen	P.o. (50 mg/kg) in mice. Drug levels measured at T_{max}.	Brain / plasma concentration ratio: Ibuprofen: 0.03 *R*-flurbiprofen: 0.04 Indomethacin: 0.01 Naproxen: 0.03 Sulindac sulfide: 0.006 Mefanamic acid: 0.03 Fenoprofen: 0.03 Methylflurbiprofen: 0.02	(Stock *et al.* 2006)
Indomethacin	P.o. (3.5 mg/kg) in rats, blood and brain samples collected 2-24 h after administration.	Brain / plasma concentration ratio: 0.02 AUC_{brain} / AUC_{plasma} ratio: 0.03	(Dvir *et al.* 2006)
Indomethacin	P.o. and i.v. infusion (3.5 mg/kg) in rats, blood and brain samples collected 8 h after administration.	Brain / plasma concentration ratio: P.o.: 0.03 I.v.: 0.08	(Dahan and Hoffman 2007)

[a] Calculated from data shown in ref.

 With the exception of the occasional case studies of fatal overdoses of NSAIDs (Kunsman and Rohrig 1993), the CNS permeation of NSAIDs in humans has been studied only by measuring the CSF concentration of the drug after a single-dose administration in adults (Table 2.4). Studies with indomethacin (Bannwarth *et al*. 1990), ketoprofen (Netter *et al*. 1985), ibuprofen (Bannwarth *et al*. 1995) and ketorolac (Rice *et al*. 1993) as well as experiments with COX2-selective agents, celecoxib, rofecoxib and valdecoxib (Dembo *et al*. 2005) demonstrate that these drugs enter the CSF. The average CSF to total plasma concentration ratio or the CSF to total plasma AUC ratio of indomethacin, ketoprofen, ibuprofen and ketorolac ranges from 0.00046 to 0.015, and the peak CSF concentration is achieved 2-4 hours after intramuscular or oral administration. Interestingly, rofecoxib exhibits higher maximum CSF to total plasma concentration ratio than the other coxibs or non-selective NSAIDs. However, when CNS penetration was evaluated based on CSF to unbound drug concentration ratio, no differences were found between different coxibs (Dembo *et al*. 2005).

 In general, studying the CSF concentrations of NSAIDs is rather difficult. Firstly, the CSF compartment is difficult to access and for ethical reasons, usually only one sample is collected from each patient. This kind of protocol is not optimal for studying the pharmacokinetics of compounds, but it is generally believed to produce valuable data about the CSF permeation of drugs (Sheiner 1984, Fowler and Dawes 1985). Secondly, the permeation of NSAIDs into the CSF is limited which places major demands on the analytical methods to be used.

43

Table 2.4. Experimental data on CNS permeation of NSAIDs in humans.

NSAID	Outcome					Ref.
	Mean CSF / plasma concentration ratio[a]		Maximun CSF/ plasma concentration ratio		C_{max} and t_{max} in CSF	
	Plasma, total	Plasma, unbound	Plasma, total	Plasma, unbound		
Indomethacin 50 mg i.m., plasma sampling 0.5-12 h post-dose	0.0074	1.44	0.029 (7 h post-dose) (0.012[d])	4.27 (4 h post-dose) (2.90[f])	11.9 ng/ml (5.1 ng/ml[d]) 2 h	(Bannwarth *et al*. 1990)
Ketoprofen 100 mg i.m., plasma sampling 0.25-13 h post-dose	0.011	0.81	0.06 (13 h post-dose) (0.029[e])	2.50 (5 h post-dose) (n=1)	18 ng/ml (14 ng/ml[f]) 4 h	(Netter *et al*. 1985)
Ketorolac 90 mg i.m., plasma sampling 1-4.5 h post-dose	0.00046	0.057	0.0018 (4.5 h post-dose)	0.23 (4.5 h post-dose)	5.7 ng/ml 2.5 h	(Rice *et al*. 1993)
Celecoxib 200 mg p.o.[b]	-	1.5 – 1.7 for all coxibs[g]	0.008[c]	-	2.4 ng/ml[c] 4.3 h[c]	(Dembo *et al*. 2005)
Rofecoxib 50 mg p.o.[b]	-		0.20[c]	-	56.6 ng/ml[c] 4.4 h[c]	
Valdecoxib 40 mg p.o.[b] Plasma sampling 0.5-10 h post-dose	-		0.040[c] (6 h post-dose)	-	9.7 ng/ml[c] 4.8 h[c]	

	Mean AUC_{CSF} / AUC_{plasma} ratio			
	Plasma, total	Plasma, unbound		
Ibuprofen 800 mg p.o., plasma sampling 0.5-8 h post-dose	R-ibuprofen: 0.009 S-ibuprofen: 0.015	R-ibuprofen: 3.6 S-ibuprofen: 3.3	R-ibuprofen: 168 ng/ml S-ibuprofen: 315 ng/ml 3 h	(Bannwarth *et al*. 1995)

[a] Calculated from data shown in ref.
[b] All three drugs dosed together.
[c] .Mean of nine subjects.
[d] Mean of four subjects at the same time point.
[e] Mean of six subjects.
[f] Mean of three subjects.
[g] Individual data not provided

2.2.1.2 Interactions with endogenous transport mechanisms

It has been suggested that NSAIDs interact with various transport mechanisms in the body. For instance, it is known that NSAIDs can interact with OATs expressed in *Xenopus laevis* oocytes (Apiwattanakul *et al*. 1999, Mulato *et al*. 2000). At the level of the BBB and the BCSFB, there might also be interactions between NSAIDs and transporters for organic anions.

Deguchi *et al*. (2000) have studied the brain delivery of ketoprofen and its triglyceride prodrug using a variety of *in vivo* and *in situ* methods in mice. One interesting observation was that ketoprofen may be transported from the brain back to the blood circulation via the efflux system for organic anions. In addition, Ohtsuki *et al*. (2002) have discovered that ketoprofen is able to inhibit significantly indoxyl sulfate uptake by OAT3 in a *Xenopus* oocyte expression system. This suggests that ketoprofen may be a substrate for this transport protein. Consequently, it is possible that the apparently poor CNS distribution of ketoprofen is partly due to efflux mechanisms at the blood-CNS barrier. *In situ* brain perfusion studies with rats have also indicated that the brain uptake of ibuprofen is partly carrier-mediated since the BBB ibuprofen transport was saturable and could be inhibited by indomethacin (Parepally *et al*. 2006).

2.2.2 Peripheral and central actions of non-steroidal anti-inflammatory drugs

NSAIDs are used as anti-inflammatory, analgesic and antipyretic agents. They are indicated to treat mild or moderate pain following injury or disease. They also have an important role in treating chronic pain states in e.g. arthritis (Burke *et al*. 2006). In severe pain, e.g. after surgery, they are advantageous when combined with opioids (Kokki 2003). NSAIDs are believed to act mainly through inhibition of prostaglandin synthesis (Vane 1971). A key enzyme in the synthesis of prostaglandins is prostaglandin endoperoxide G/H synthase, more commonly called cyclooxygenase (COX) (Smyth *et al*. 2006). There are at least two forms of COX, COX1 and COX2, which have different localisations and functions. It is believed that COX2 is induced in inflammatory conditions whereas COX1 is a constitutive enzyme found in almost all cells. However, COX2 is also expressed constitutively in some tissues, including the brain (Kaufmann *et al*. 1997), and it seems that both isoenzymes have physiological and pathological roles (Loftin *et al*. 2002). Most clinically used NSAIDs inhibit both isoenzymes, only the degree of selectivity varies.

2.2.2.1 Antihyperalgesic effects

Nociceptors or pain receptors are receptors for injurious or painful stimuli. In inflammation, various inflammatory mediators interact with nociceptors causing pain. Prostaglandins can act as sensitizing agents for enhancing the nociceptive properties of these inflammatory

mediators, and this is the reason why inhibition of prostaglandin synthesis is able to evoke an antinociceptive effect. The antinociceptive effect of NSAIDs can be described better as being antihyperalgesic rather than as analgesic because NSAIDs, through inhibiting prostaglandin formation, reverse the increase in response to noxious stimuli and the appearance of spontaneous activity in the nociceptors, the hallmarks of inflammatory pain, rather than elevating the normal pain threshold (Burian and Geisslinger 2005).

The antinociceptive effects of NSAIDs are usually believed to be mediated mainly in peripheral tissues. However, it has been claimed that NSAIDs act also at the central level (Ossipov *et al*. 2000, Malmberg and Yaksh 1992, Daher and Tonussi 2003). The central mechanism of action of NSAIDs has been established e.g. by administering NSAIDs directly into the CNS in animal models of inflammatory pain. For example, Malmberg and Yaks (1992) have studied the antinociceptive potency and efficacy of various NSAIDs after intrathecal administration. According to their studies, NSAIDs displayed dose-dependent antinociceptive actions in the rat after intrathecal administration which they proposed was evidence for the existence of a central mode of action. The potencies varied considerably between different NSAIDs. NSAIDs also reduced nociceptive behaviour after intraperitoneal administration, but the agents were 100 to 1000 times more potent after spinal than after intraperitoneal administration. For instance, indomethacin had ID_{50} values of 1.9 nmol after intrathecal delivery compared to 2600 nmol after intraperitoneal administration. The ID_{50} value represents the dose resulting in 50% inhibition of the control response in the formalin test.

In conclusion, according to our present understanding, the antihyperalgesic effect of NSAIDs takes place both at the peripheral and central levels. The relative contribution of the two components depends on the particular NSAID used, the site of drug delivery and how the drug reaches its site of action (Burian and Geisslinger 2005). With respect to the central actions of NSAIDs, the drug levels in brain or spinal tissue are critical. However, at present, there is inadequate data on the CNS distribution of NSAIDs after therapeutic administration. Accordingly, it is not understood how the drug levels in the CNS after experimental local administration directly into the CNS and after therapeutic systemic administration correspond to each other.

2.2.2.2 Alzheimer's disease and non-steroidal anti-inflammatory drugs

AD is the most common cause of dementia in the elderly. It is a progressive neurodegenerative disorder which eventually causes death. It is traditionally characterized neuropathologically by the presence of senile plaques, neurofibrillary tangles, cerebral amyloid angiopathy and loss of neurons (Selkoe 2001). After reports that people suffering from inflammatory diseases, such as rheumatoid arthritis, had a lower incidence of AD, there

has been interest towards the potential of using NSAIDs as a means to achieve AD prevention.

AD is a disease which has been extensively investigated and for example, some genetic factors underlying the disease have been identified. These include mutations in β-amyloid precursor protein and presenillins which are responsible for the rather infrequent familial forms of AD, and the presence of the apolipoprotein ε4 allele, which is a major risk factor for the disease (Selkoe 2001). However, the exact pathogenesis of AD is not completely understood. It is, for example, recognized that amyloid β (Aβ) peptide, a primary component of senile plaques, has a crucial role in the development of AD pathology but whether it actually is causing the disease, is far from clear. It is also known that chronic neuroinflammation occurs in the AD brain. At the cellular and molecular levels, inflammation can be seen as the presence of activated microglia and reactive astrocytes and altered concentrations of various pro- and anti-inflammatory mediators (Akiyama *et al.* 2000).

The onset of AD is insidious and it is generally believed that the disease begins decades before any clinical symptoms are detected. It is evident that this first, latent phase of the disease would represent the best time to initiate some kind of prevention or treatment strategy (Zandi and Breitner 2001). To date, several epidemiological studies have provided clear evidence that the use of NSAIDs may reduce the risk or delay the onset of AD (Etminan *et al*. 2003, Szekely *et al*. 2004, Vlad *et al*. 2008). This epidemiological evidence together with the recognition that neuroinflammation may have a role in the pathogenesis of AD and the emerging need for preventive strategies against the disease has represented the rationale behind a series of laboratory studies with NSAIDs. Despite much work done with both cell cultures and animal models (Townsend and Pratico 2005, Gasparini *et al*. 2004), the exact mechanisms of action by which NSAIDs may be able to modify AD have not yet been described. In addition to COX-inhibition, it has been suggested that the beneficial effects of some NSAIDs in AD could be mediated by interactions with nuclear factor-κB and peroxisome proliferator-activated receptor-γ and subsequent reduction in inflammatory processes, as well as direct anti-amyloid mechanisms. To date, only a few randomised, controlled clinical trials have been conducted with NSAIDs but unfortunately, they have not been encouraging. For instance, although a small pilot trial with indomethacin did show positive results (Rogers *et al*. 1993), more recent studies with larger populations investigating the effect of rofecoxib and naproxen in combating the progress of AD produced negative results (Reines *et al*. 2004, Aisen *et al*. 2003).

According to the *in vitro* data, studies with transgenic animal models and clinical trials, it has been proposed that only some of the NSAIDs have AD modifying properties. For instance, it has been argued that only COX-nonselective NSAIDs rather than the COX-selective agents have beneficial effects against AD (Townsend and Pratico 2005). In addition, NSAIDs may be efficient in preventing rather than treating the disease. In order to

demonstrate the usefulness of NSAIDs in treating or preventing AD, prospective, long-term prevention trials will be required. To attain full advantage from such a study, several aspects in the study design will have to be taken into account - for example COX-selectivity and anti-amyloidogenic properties of the drug, brain penetration properties of the drug, the dose, since COX-independent mechanisms are only achieved at high drug concentration *in vitro*, duration of the treatment, as it has been suggested that the exposure to NSAIDs should be at least 2 years in duration in order to produce beneficial effects (Stewart *et al*. 1997, in t' Veld *et al*. 2001), and the stage of the disease at the onset of treatment, since people already diagnosed with AD may have suffered neuronal damage that is too advanced to obtain any clinical benefit from NSAIDs.

48

2.3 References

Abbott, N. J. Evidence for bulk flow of brain interstitial fluid: significance for physiology and pathology. Neurochem Int 45: 545-52, 2004.

Abbott, N. J., Ronnback, L., Hansson, E. Astrocyte-endothelial interactions at the blood-brain barrier. Nat Rev Neurosci 7**:** 41-53, 2006.

Abraham, M. H., Platts, J. A. Physicochemical factors that influence brain uptake. In Begley, D. J., Bradbury, M. W. & Kreuter, J. (Eds.) The blood-brain barrier and drug delivery to the CNS. New York, Marcel Dekker, Inc, 2000.

Adkison, K. D., Shen, D. D. Uptake of valproic acid into rat brain is mediated by a medium-chain fatty acid transporter. J Pharmacol Exp Ther 276**:** 1189-200, 1996.

Aisen, P. S., Schafer, K. A., Grundman, M., Pfeiffer, E., Sano, M., Davis, K. L., Farlow, M. R., Jin, S., Thomas, R. G., Thal, L. J. Effects of rofecoxib or naproxen vs placebo on Alzheimer disease progression: a randomized controlled trial. Jama 289: 2819-26, 2003.

Akiyama, H., Barger, S., Barnum, S., Bradt, B., Bauer, J., Cole, G. M., Cooper, N. R., Eikelenboom, P., Emmerling, M., Fiebich, B. L., Finch, C. E., Frautschy, S., Griffin, W. S., Hampel, H., Hull, M., Landreth, G., Lue, L., Mrak, R., Mackenzie, I. R., McGeer, P. L., O'Banion, M. K., Pachter, J., Pasinetti, G., Plata-Salaman, C., Rogers, J., Rydel, R., Shen, Y., Streit, W., Strohmeyer, R., Tooyoma, I., Van Muiswinkel, F. L., Veerhuis, R., Walker, D., Webster, S., Wegrzyniak, B., Wenk, G., Wyss-Coray, T. Inflammation and Alzheimer's disease. Neurobiol Aging 21**:** 383-421, 2000.

Anderson, B. D. Prodrugs for improved CNS delivery. Adv Drug Deliv Rev 19**:** 171-202, 1996.

Anderson, B. D., Galinsky, R. E., Baker, D. C., Chi, S. C., Hoesterey, B. L., Morgan, M. E., Murakami, K., Mitsuya, H. Approaches toward the optimization of CNS uptake of anti-AIDS agents. J Control Release 19**:** 219-230, 1991.

Apiwattanakul, N., Sekine, T., Chairoungdua, A., Kanai, Y., Nakajima, N., Sophasan, S., Endou, H. Transport properties of nonsteroidal anti-inflammatory drugs by organic anion transporter 1 expressed in Xenopus laevis oocytes. Mol Pharmacol 55: 847-54, 1999.

Austin, R. P., Barton, P., Cockroft, S. L., Wenlock, M. C., Riley, R. J. The influence of nonspecific microsomal binding on apparent intrinsic clearance, and its prediction from physicochemical properties. Drug Metab Dispos 30**:** 1497-503, 2002.

Bannwarth, B., Lapicque, F., Pehourcq, F., Gillet, P., Schaeverbeke, T., Laborde, C., Dehais, J., Gaucher, A., Netter, P. Stereoselective disposition of ibuprofen enantiomers in human cerebrospinal fluid. Br J Clin Pharmacol 40: 266-9, 1995.

Bannwarth, B., Netter, P., Lapicque, F., Pere, P., Thomas, P., Gaucher, A. Plasma and cerebrospinal fluid concentrations of indomethacin in humans. Relationship to analgesic activity. Eur J Clin Pharmacol 38: 343-6, 1990.

Barbato, F., La Rotonda, M. I., Quaglia, F. Interactions of nonsteroidal antiinflammatory drugs with phospholipids: comparison between octanol/buffer partition coefficients and chromatographic indexes on immobilized artificial membranes. J Pharm Sci 86**:** 225-9, 1997.

Battaglia, G., La Russa, M., Bruno, V., Arenare, L., Ippolito, R., Copani, A., Bonina, F., Nicoletti, F. Systemically administered D-glucose conjugates of 7-chlorokynurenic acid are centrally available and exert anticonvulsant activity in rodents. Brain Res 860**:** 149-56, 2000.

Begley, D. J. Delivery of therapeutic agents to the central nervous system: the problems and the possibilities. Pharmacol Ther 104**:** 29-45, 2004.

49

Bickel, U. How to measure drug transport across the blood-brain barrier. NeuroRx 2: 15-26, 2005.

Bodor, N., Buchwald, P. Barriers to remember: brain-targeting chemical delivery systems and Alzheimer's disease. Drug Discov Today, 7**:** 766-74, 2002.

Bonate, P. L. Animal models for studying transport across the blood-brain barrier. J Neurosci Methods 56**:** 1-15, 1995.

Bonina, F. P., Arenare, L., Ippolito, R., Boatto, G., Battaglia, G., Bruno, V., de Caprariis, P. Synthesis, pharmacokinetics and anticonvulsant activity of 7-chlorokynurenic acid prodrugs. Int J Pharm 202**:** 79-88, 2000.

Brodie, B. B., Kurz, H., Schanker, L. S. The importance of dissociation constant and lipid-solubility in influencing the passage of drugs into the cerebrospinal fluid. J Pharmacol Exp Ther 130: 20-25, 1960.

Burian, M., Geisslinger, G. COX-dependent mechanisms involved in the antinociceptive action of NSAIDs at central and peripheral sites. Pharmacol Ther 107**:** 139-54, 2005.

Burke, A., Smyth, E. M., FitzGerald, G. A. Analgesic-Antipyretic Agents; Pharmacotherapy of Gout. In Brunton, L. L., Lazo, J. S. & Parker, K. L. (Eds.) Goodman & Gilman's The Pharmacological Basis of Therapeutics. 11th ed. New York, McGraw-Hill Companies, Inc, 2006.

Buxton, I. L. O. Pharmacokinetics ans Pharmacodynamics: The Dynamics of Drug Absorption, Distribution, Action, and Elimination. In Brunton, L. L., Lazo, J. S., Parker, K. L. (Eds.) Goodman & Gilman's The Pharmacological Basis of Therapeutics. 11th ed. New York, McGraw-Hill Companies, Inc, 2006.

Chikhale, E. G., Burton, P. S., Borchardt, R. T. The effect of verapamil on the transport of peptides across the blood-brain barrier in rats: kinetic evidence for an apically polarized efflux mechanism. J Pharmacol Exp Ther 273: 298-303, 1995.

Cisternino, S., Mercier, C., Bourasset, F., Roux, F., Scherrmann, J. M. Expression, up-regulation, and transport activity of the multidrug-resistance protein Abcg2 at the mouse blood-brain barrier. Cancer Res 64: 3296-301, 2004.

Clark, D. E. In silico prediction of blood-brain barrier permeation. Drug Discov Today 8: 927-33, 2003.

Craft, J. C., Feldman, W. E., Nelson, J. D. Clinicopharmacological evaluation of amoxicillin and probenecid against bacterial meningitis. Antimicrob Agents Chemother 16: 346-52, 1979.

Dahan, A., Hoffman, A. Mode of administration-dependent brain uptake of indomethacin: sustained systemic input increases brain influx. Drug Metab Dispos 35**:** 321-4, 2007.

Daher, J. B., Tonussi, C. R. A spinal mechanism for the peripheral anti-inflammatory action of indomethacin. Brain Res 962: 207-12, 2003.

Davson, H., Segal, M. B. Physiology of the CSF and the blood-brain barriers, New York, CRC Press, Inc, 1996.

de Boer, A. G., Gaillard, P. J. Drug targeting to the Brain. Ann Rev Pharmacol Toxicol 47: 323-55, 2007.

de Lange, E. C. M., de Bock, G., Schinkel, A. H., de Boer, A. G., Breimer, D. D. BBB transport and P-glycoprotein functionality mdr1a(-/-) and wild-type mice. Total brain versus microdialysis concentration profiles of rhodamine-123. Pharm Res 15: 1657-65, 1998.

de Lange, E. C. M., de Boer, B. A. G., Breimer, D. D. Microdialysis for the pharmacokinetic analysis of drug transport to the brain. Adv Drug Deliv Rev 36: 211-27, 1999.

de Lange, E. C. M. Potential role of ABC-transporters as a detoxification system at the blood-CSF barrier. Adv Drug Deliv Rev 56: 1793-1809, 2004.

de Lange, E. C. M., Danhof, M. Considerations in the use of cerebrospinal fluid pharmacokinetics to predict brain target concentrations in the clinical setting: implications of the barriers between blood and brain. Clin Pharmacokinet 41: 691-703, 2002.

Deguchi, Y., Inabe, K., Tomiyasu, K., Nozawa, K., Yamada, S. , Kimura, R. Study on brain interstitial fluid distrubution and blood-brain barrier transport of baclofen in rats by microdialysis. Pharm Res 12: 1838-44, 1995.

Deguchi, Y., Nozawa, K., Yamada, S., Yokoyama, Y., Kimura, R. Quantitative evaluation of brain distribution and blood-brain barrier efflux transport of probenecid in rats by microdialysis: possible involvement of the monocarboxylic acid transport system. J Pharmacol Exp Ther 280**:** 551-60, 1997.

Deguchi, Y., Hayashi, H., Fujii, S., Naito, T., Yokoyama, Y., Yamada, S., Kimura, R. Improved brain delivery of a nonsteroidal anti-inflammatory drug with a synthetic glyceride ester: a preliminary attempt at a CNS drug delivery system for the therapy of Alzheimer's disease. J Drug Target 8: 371-81, 2000.

Dembo, G., Park, S. B., Kharasch, E. D. Central nervous system concentrations of cyclooxygenase-2 inhibitors in humans. Anesthesiology 102: 409-15, 2005.

Dobson, P. D., Kell, D. B. Carrier-mediated cellular uptake of pharmaceutical drugs: an exception or the rule? Nat Rev Drug Discov 7: 205-20, 2008.

Dubey, R. K., McAllister, C. B., Inoue, M., Wilkinson, G. R. Plasma binding and transport of diazepam across the blood-brain barrier. No evidence for in vivo enhanced dissociation. J Clin Invest 84: 1155-9, 1989.

Dvir, E., Friedman, J. E., Lee, J. Y., Koh, J. Y., Younis, F., Raz, S., Shapiro, I., Hoffman, A., Dahan, A., Rosenberg, G., Angel, I., Kozak, A., Duvdevani, R. A novel phospholipid derivative of indomethacin, DP-155 [mixture of 1-steroyl and 1-palmitoyl-2-{6-[1-(p-chlorobenzoyl)-5-methoxy-2-methyl-3-indolyl acetamido]hexanoyl}-sn-glycero-3-phosophatidyl [corrected] choline], shows superior safety and similar efficacy in reducing brain amyloid beta in an Alzheimer's disease model. J Pharmacol Exp Ther 318**:** 1248-56, 2006.

Eriksen, J. L., Sagi, S. A., Smith, T. E., Weggen, S., Das, P., McLendon, D. C., Ozols, V. V., Jessing, K. W., Zavitz, K. H., Koo, E. H., Golde, T. E. NSAIDs and enantiomers of flurbiprofen target gamma-secretase and lower Abeta 42 in vivo. J Clin Invest 112: 440-9, 2003.

Etminan, M., Gill, S., Samii, A. Effect of non-steroidal anti-inflammatory drugs on risk of Alzheimer's disease: systematic review and meta-analysis of observational studies. BMJ 327**:** 128-32, 2003.

Faraci, F. M., Kinzenbaw, D., Heistad, D. D. Effect on endogenous vasopressin on blood flow to choroid plexus during hypoxia and intracranial hypertension. Am J Physiol 266: H393-H398, 1994.

Fellner, S., Bauer, B., Miller, D. S., Schaffrik, M., Fankhanel, M., Spruss, T., Bernhardt, G., Graeff, C., Farber, L., Gschaidmeier, H., Buschauer, A., Fricker, G. Transport of paclitaxel (Taxol) across the blood-brain barrier in vitro and in vivo. J Clin Invest 110: 1309-18, 2002.

Fenstermacher, J. D. Pharmacology of the Blood-Brain Barrier. In Neuwelt, E. A. (Ed.) Implications of the Blood-Brain Barrier and Its Manipulation. New York, Plenum Medical Book Company, 1989.

Foster, R. T., Jamali, F. Stereoselective pharmakocinetics of ketoprofen in the rat. Drug Metab Dispos 16: 623-6, 1988.

Fowler, P. D., Dawes, P. T. Osmosin: its effect on plasma and synovial fluid kinetics of indomethacin. Eur J Clin Pharmacol 27: 689-91, 1985.

Frijlink, H. W., Franssen, E. J., Eissens, A. C., Oosting, R., Lerk, C. F., Meijer, D. K. The effects of cyclodextrins on the disposition of intravenously injected drugs in the rat. Pharm Res 8**:** 380-4, 1991.

Galinsky, R. E., Flaharty, K. K., Hoesterey, B. L., Anderson, B. D. Probenecid enhances central nervous system uptake of 2',3'-dideoxyinosine by inhibiting cerebrospinal fluid efflux. J Pharmacol Exp Ther 257: 972-8, 1991.

Gasparini, L., Ongini, E., Wenk, G. Non-steroidal anti-inflammatory drugs (NSAIDs) in Alzheimer's disease: old and new mechanisms of action. J Neurochem 91**:** 521-36, 2004.

Ghersi-Egea, J. F., Leininger-Muller, B., Cecchelli, R., Fenstermacher, J. D. Blood-brain interfaces: relevance to cerebral drug metabolism. Toxicol Lett 82-83**:** 645-53, 1995.

Greig, N. H. Drug Delivery to the Brain by Blood-Brain Barrier Circumvention and Drug Modification. In Neuwelt, E. A. (Ed.) Implications of the Blood-Brain Barrier and Its Manipulation. New York, Plenum Medical Book Company, 1989.

Greig, N. H., Daly, E. M., Sweeney, D. J., Rapoport, S. I. Pharmacokinetics of chlorambucil-tertiary butyl ester, a lipophilic chlorambucil derivative that achieves and maintains high concentrations in brain. Cancer Chemother Pharmacol 25: 320-5, 1990.

Gumbleton, M., Audus, K. L. Progress and limitations in the use of in vitro cell cultures to serve as a permeability screen for the blood-brain barrier. J Pharm Sci 90: 1681-98, 2001.

Gynther, M., Laine, K., Ropponen, J., Leppänen, J., Mannila, A., Nevalainen, T., Savolainen, J., Järvinen, T., Rautio, J. Large Neutral Amino Acid Transporter Enables Brain Drug Delivery via Prodrugs. J Med Chem 51: 932-6, 2008.

Hagenbuch, B., Meier, P. J. Organic anion transporting polypeptides of the OATP/SLC21 family: phylogenetic classification as OATP/SLCO superfamily, new nomenclature and molecular/functional properties. Pflugers Arch – Eur J Physiol 447: 653-65, 2004.

Hammarlund-Udenaes, M. The use of microdialysis in CNS drug delivery studies. Pharmacokinetic perspectives and results with analgesics and antiepileptics. Adv Drug Deliv Rev 45: 283-94, 2000.

Hokari, M., Wu, H. Q., Schwarcz, R., Smith, Q. R. Facilitated brain uptake of 4-chlorokynurenine and conversion to 7-chlorokynurenic acid. Neuroreport 8: 15-8, 1996.

Huber, J. D., Witt, K. A., Hom, S., Egleton, R. D., Mark, K. S., Davis, T. P. Inflammatory pain alters blood-brain barrier permeability and tight junctional protein expression. Am J Physiol Heart Circ Physiol 280: H1241-8, 2001.

Hucker, H. B., Zacchei, A. G., Cox, S. V., Brodie, D. A., Cantwell, N. H. R. Studies on the absorption, distribution and excretion of indomethacin in various species. JPET 153: 237-249, 1966.

Huynh, G. H., Deen, D. F., Szoka, F. C., Jr. Barriers to carrier mediated drug and gene delivery to brain tumors. J Control Release 110**:** 236-59, 2006.

in t' Veld, B. A., Ruitenberg, A., Hofman, A., Launer, L. J., van Duijn, C. M., Stijnen, T., Breteler, M. M., Stricker, B. H. Nonsteroidal antiinflammatory drugs and the risk of Alzheimer's disease. N Engl J Med 345: 1515-21, 2001.

Ishizaki, T., Sasaki, T., Suganuma, T., Horai, Y., Chiba, K., Watanabe, M., Asuke, W., Hoshi, H. Pharmacokinetics of ketoprofen following single oral, intramuscular and rectal doses and after repeated oral administration. Eur J Clin Pharmacol 18: 407-14, 1980.

Jedlitschky, G., Leier, I., Buchholz, U., Barnouin, K., Kurz, G., Keppler, D. Transport of glutathione, glucuronate, and sulfate conjugates by the MRP gene-encoded conjugate export pump. Cancer Res 56: 988-94, 1996.

Johanson, C. E., Duncan, J. A., Stopa, E. G., Baird, A. Enhanced prospects for drug delivery and brain targeting by the choroid plexus-CSF route. Pharm Res 22: 1011-37, 2005.

Johnson, M. D., Anderson, B. D. Localization of purine metabolizing enzymes in bovine brain microvessel endothelial cells: an enzymatic blood-brain barrier for dideoxynucleosides? Pharm Res 13: 1881-6, 1996.

Jolliet, P., Simon, N., Bree, F., Urien, S., Pagliara, A., Carrupt, P. A., Testa, B., Tillement, J. P. Blood-to-brain transfer of various oxicams: effects of plasma binding on their brain delivery. Pharm Res 14 650-6, 1997.

Kakee, A., Terasaki, T., Sugiyama, Y. Selective brain to blood efflux transport of para-aminohippuric acid across the blood-brain barrier: in vivo evidence by use of the brain efflux index method. J Pharmacol Exp Ther 283: 1018-25, 1997.

Kalvass, J. C., Pollack, G. M. Kinetic considerations for the quantitative assessment of efflux activity and inhibition: implications for understanding and predicting the effects of efflux inhibition. Pharm Res 24: 265-76, 2007.

Kaufmann, W. E., Andreasson, K. I., Isakson, P. C., Worley, P. F. Cyclooxygenases and the central nervous system. Prostaglandins 54: 601-24, 1997.

Keep, R. F., Jones, H. C. A morphometric study on the development of the lateral ventricle choroid plexus, choroid plexus capillaries and ventricular ependyma in the rat. Brain Res Dev Brain Res 56: 47-53, 1990.

Kikuchi, R., Kusuhara, H., Sugiyama, D., Sugiyama, Y. Contribution of organic anion transporter 3 (Slc22a8) to the elimination of p-aminohippuric acid and benzylpenicillin across the blood-brain barrier. J Pharmacol Exp Ther 306: 51-8, 2003.

Koepsell, H., Endou, H. The SLC22 drug transporter family. Pflugers Arch – Eur J Physiol 447: 666-76, 2004.

Kokki, H. Nonsteroidal anti-inflammatory drugs for postoperative pain: a focus on children. Paediatr Drugs 5: 103-23, 2003.

Kragh-Hansen, U., Chuang, V. T., Otagiri, M. Practical aspects of the ligand-binding and enzymatic properties of human serum albumin. Biol Pharm Bull 25: 695-704, 2002.

Kukar, T., Murphy, M. P., Eriksen, J. L., Sagi, S. A., Weggen, S., Smith, T. E., Ladd, T., Khan, M. A., Kache, R., Beard, J., Dodson, M., Merit, S., Ozols, V. V., Anastasiadis, P. Z., Das, P., Fauq, A., Koo, E. H., Golde, T. E. Diverse compounds mimic Alzheimer disease-causing mutations by augmenting Abeta42 production. Nat Med 11: 545-50, 2005.

Kunsman, G. W., Rohrig, T. P. Tissue distribution of ibuprofen in a fatal overdose. Am J Forensic Med Pathol 14: 48-50, 1993.

Kusuhara, H., Sekine, T., Utsunomiya-Tate, N., Tsuda, M., Kojima, R., Cha, S. H., Sugiyama, Y., Kanai, Y., Endou, H. Molecular cloning and characterization of a new multispecific organic anion transporter from rat brain. J Biol Chem 274: 13675-80, 1999.

Kusuhara, H., Sugiyama, Y. Efflux transport systems for organic anions and cations at the blood-CSF barrier. Adv Drug Deliv Rev 56: 1741-63, 2004.

Kusuhara, H., Sugiyama, Y. Active efflux across the blood-brain barrier: role of the solute carrier family. NeuroRx 2: 73-85, 2005.

Lee, E. J., Williams, K., Day, R., Graham, G., Champion, D. Stereoselective disposition of ibuprofen enantiomers in man. Br J Clin Pharmacol 19: 669-74, 1985.

Leggas, M., Adachi, M., Scheffer, G. L., Sun, D., Wielinga, P., Du, G., Mercer, K. E., Zhuang, Y., Panetta, J. C., Johnston, B., Scheper, R. J., Stewart, C. F., Schuetz, J. D. Mrp4 confers resistance to topotecan and protects the brain from chemotherapy. Mol Cell Biol 24: 7612-21, 2004.

Leisen, C., Langguth, P., Herbert, B., Dressler, C., Koggel, A., Spahn-Langguth, H. Lipophilicities of baclofen ester prodrugs correlate with affinities to the ATP-dependent efflux pump P-glycoprotein: relevance for their permeation across the blood-brain barrier? Pharm Res 20: 772-8, 2003.

Letrent, S. P., Pollack, G. M., Brouwer, K. R., Brouwer, K. L. Effects of a potent and specific P-glycoprotein inhibitor on the blood-brain barrier distribution and antinociceptive effect of morphine in the rat. Drug Metab Dispos 27: 827-34, 1999.

Levin, V. A. Relationship of octanol/water partition coefficient and molecular weight to rat brain capillary permeability. J Med Chem 23: 682-4, 1980.

Lin, J. H., Yamazaki, M. Clinical relevance of P-glycoprotein in drug therapy. Drug Metab Rev 35: 417-54, 2003.

Liu, X., Smith, B. J., Chen, C., Callegari, E., Becker, S. L., Chen, X., Cianfrogna, J., Doran, A. C., Doran, S. D., Gibbs, J. P., Hosea, N., Liu, J., Nelson, F. R., Szewc, M. A., Van Deusen, J. Use of a physiologically based pharmacokinetic model to study the time to reach brain equilibrium: an experimental analysis of the role of blood-brain barrier permeability, plasma protein binding, and brain tissue binding. J Pharmacol Exp Ther 313: 1254-62, 2005.

Liu, X., Van Natta, K., Yeo, H., Vilenski, O., Weller, P. E., Worboys, P. D., Monshouwer, M. Unbound drug concentrations in brain homogenate and cerebral spinal fluid at steady state as surrogate for unbound concentration in brain interstitial fluid. Drug Metabol Dispos 37:787-93, 2008

Loftin, C. D., Tiano, H. F., Langenbach, R. Phenotypes of the COX-deficient mice indicate physiological and pathophysiological roles for COX-1 and COX-2. Prostaglandins Other Lipid Mediat 68-69: 177-85, 2002.

Lundquist, S., Renftel, M., Brillault, J., Fenart, L., Cecchelli, R., Dehouck, M. P. Prediction of drug transport through the blood-brain barrier in vivo: a comparison between two in vitro cell models. Pharm Res 19: 976-81, 2002.

Malhotra, B. K., Lemaire, M., Sawchuk, R. J. Investigation of the distribution of EAB 515 to cortical ECF and CSF in freely moving rats using microdialysis. Pharm Res 11: 1223-32, 1994.

Malmberg, A. B., Yaksh, T. L. Antinociceptive actions of spinal nonsteroidal anti-inflammatory agents on the formalin test in the rat. J Pharmacol Exp Ther 263: 136-46, 1992.

Mandula, H., Parepally, J. M., Feng, R., Smith, Q. R. Role of site-specific binding to plasma albumin in drug availability to brain. J Pharmacol Exp Ther 317: 667-75, 2006.

Manfredini, S., Pavan, B., Vertuani, S., Scaglianti, M., Compagnone, D., Biondi, C., Scatturin, A., Tanganelli, S., Ferraro, L., Prasad, P., Dalpiaz, A. Design, synthesis and activity of ascorbic acid prodrugs of nipecotic, kynurenic and diclophenamic acids, liable to increase neurotropic activity. J Med Chem 45: 559-62, 2002.

Matoga, M., Pehourcq, F., Lagrange, F., Fawaz, F., Bannwarth, B. Influence of a polymeric formulation of ketoprofen on its diffusion into cerebrospinal fluid in rats. J Pharm Biomed Anal 27: 881-8, 2002.

Mayer, U., Wagenaar, E., Beijnen, J. H., Smit, J. W., Meijer, D. K., van Asperen, J., Borst, P., Schinkel, A. H. Substantial excretion of digoxin via the intestinal mucosa and prevention of long-term digoxin accumulation in the brain by the mdr 1a P-glycoprotein. Br J Pharmacol 119: 1038-44, 1996.

Minn, A., El-Bachá, R. D. S., Bayol-Denizot, C., Lagrange, P., Suleman, F. G., Gradinaru, D. Drug Metabolism in the Brain: Benefits and Risks. In Begley, D. J., Bradbury, M. W., Kreuter, J. (Eds.) The Blood-Brain Barrier and Drug Delivery to the CNS. New York, Marcel Dekker, Inc, 2000.

Minn, A., Ghersi-Egea, J. F., Perrin, R., Leininger, B., Siest, G. Drug metabolizing enzymes in the brain and cerebral microvessels. Brain Res Brain Res Rev 16: 65-82, 1991.

Mori, S., Ohtsuki, S., Takanaga, H., Kikkawa, T., Kang, Y. S., Terasaki, T. Organic anion transporter 3 is involved in the brain-to-blood efflux transport of thiopurine nucleobase analogs. J Neurochem 90: 931-41, 2004.

Mulato, A. S., Ho, E. S., Cihlar, T. Nonsteroidal anti-inflammatory drugs efficiently reduce the transport and cytotoxicity of adefovir mediated by the human renal organic anion transporter 1. J Pharmacol Exp Ther 295: 10-5, 2000.

Netter, P., Lapicque, F., Bannwarth, B., Tamisier, J. N., Thomas, P., Royer, R. J. Diffusion of intramuscular ketoprofen into the cerebrospinal fluid. Eur J Clin Pharmacol 29: 319-21, 1985.

Nicolazzo, J. A., Charman, S. A., Charman, W. N. Methods to assess drug permeability across the blood-brain barrier. J Pharm Pharmacol 58: 281-93, 2006.

Ogawa, M., Suzuki, H., Sawada, Y., Hanano, M., Sugiyama, Y. Kinetics of active efflux via choroid plexus of beta-lactam antibiotics from the CSF into the circulation. Am J Physiol 266: R392-9, 1994.

Ohtsuki, S., Asaba, H., Takanaga, H., Deguchi, T., Hosoya, K., Otagiri, M., Terasaki, T. Role of blood-brain barrier organic anion transporter 3 (OAT3) in the efflux of indoxyl sulfate, a uremic toxin: its involvement in neurotransmitter metabolite clearance from the brain. J Neurochem 83: 57-66, 2002.

Ooie, T., Terasaki, T., Suzuki, H., Sugiyama, Y. Quantitative brain microdialysis study on the mechanisms of quinolones distribution in the central nervous system. Drug Metab Dispos 25: 784-9, 1997.

Ossipov, M. H., Jerussi, T. P., Ren, K., Sun, H., Porreca, F. Differential effects of spinal (R)-ketoprofen and (S)-ketoprofen against signs of neuropathic pain and tonic nociception: evidence for a novel mechanism of action of (R)-ketoprofen against tactile allodynia. Pain 87: 193-9, 2000.

Pardridge, W. M. Transport of small molecules through the blood-brain barrier: biology and methodology. Advanced Drug Delivery Reviews 15: 5-36, 1995.

Pardridge, W. M. Drug and gene targeting to the brain with molecular Trojan horses. Nat Rev Drug Discov 1: 131-9, 2002.

Pardridge, W. M. Blood-brain barrier drug targeting: the future of brain drug development. Mol Interv 3: 90-105, 51, 2003.

Pardridge, W. M., Triguero, D., Yang, J., Cancilla, P. A. Comparison of in vitro and in vivo models of drug transcytosis through the blood-brain barrier. J Pharmacol Exp Ther 253: 884-91, 1990.

Parepally, J. M., Mandula, H., Smith, Q. R. Brain Uptake of Nonsteroidal Anti-Inflammatory Drugs: Ibuprofen, Flurbiprofen, and Indomethacin. Pharm Res 23: 873-81, 2006.

Pehourcq, F., Matoga, M., Bannwarth, B. Diffusion of arylpropionate non-steroidal anti-inflammatory drugs into the cerebrospinal fluid: a quantitative structure-activity relationship approach. Fundam Clin Pharmacol 18: 65-70, 2004.

Polli, J. W., Jarrett, J. L., Studenberg, S. D., Humphreys, J. E., Dennis, S. W., Brouwer, K. R., Woolley, J. L. Role of P-glycoprotein on the CNS disposition of amprenavir (141W94), an HIV protease inhibitor. Pharm Res 16: 1206-12, 1999.

Polt, R., Porreca, F., Szabo, L. Z., Bilsky, E. J., Davis, P., Abbruscato, T. J., Davis, T. P., Harvath, R., Yamamura, H. I., Hruby, V. J. Glycopeptide enkephalin analogues produce analgesia in mice: evidence for penetration of the blood-brain barrier. Proc Natl Acad Sci U S A 91: 7114-8, 1994.

Rautio, J., Taipale, H., Gynther, J., Vepsäläinen, J., Nevalainen, T., Järvinen, T. In vitro evaluation of acyloxyalkyl esters as dermal prodrugs of ketoprofen and naproxen. J Pharm Sci 87: 1622-8, 1998.

Reines, S. A., Block, G. A., Morris, J. C., Liu, G., Nessly, M. L., Lines, C. R., Norman, B. A., Baranak, C. C. Rofecoxib: no effect on Alzheimer's disease in a 1-year, randomized, blinded, controlled study. Neurology 62: 66-71, 2004.

Rice, A. S., Lloyd, J., Bullingham, R. E., O'Sullivan, G. Ketorolac penetration into the cerebrospinal fluid of humans. J Clin Anesth 5: 459-62, 1993.

Rice, M. E. Ascorbate regulation and its neuroprotective role in the brain. Trends Neurosci 23**:** 209-16, 2000.

Rogers, J., Kirby, L. C., Hempelman, S. R., Berry, D. L., McGeer, P. L., Kaszniak, A. W., Zalinski, J., Cofield, M., Mansukhani, L., Willson, P., Kogan, F. Clinical trial of indomethacin in Alzheimer's disease. Neurology 43: 1609-11, 1993.

Rowland, L. P., Fink, M. E., Rubin, L. Cerebrospinal Fluid: Blood-Brain Barrier, Brain Edema, and Hydrocephalus. In Kandel, E. R., Schwartz, J. H., Jessel, T. M. (Eds.) Principles of Neural Science. 3rd. ed. New York, Elsevier, 1991.

Rowland, M., Tozer, T. N. Clinical Pharmacokinetics, Concepts and Applications, Philadelphia, Lippincott Williams, Wilkins, 1995.

Sadeque, A. J., Wandel, C., He, H., Shah, S., Wood, A. J. Increased drug delivery to the brain by P-glycoprotein inhibition. Clin Pharmacol Ther 68: 231-7, 2000.

Satterwhite, J. H., Boudinot, F. D. Effects of age and dose on the pharmacokinetics of ibuprofen in the rat. Drug Metab Dispos 19: 61-67, 1991.

Satterwhite, J. H., Boudinot, F. D. Pharmacokinetics of ketoprofen in rats: effect of age and dose. Biopharm Drug Dispos 13: 197-212, 1992.

Savolainen, J., Edwards, J. E., Morgan, M. E., McNamara, P. J., Anderson, B. D. Effects of a P-glycoprotein inhibitor on brain and plasma concentrations of anti-human immunodeficiency virus drugs administered in combination in rats. Drug Metab Dispos 30: 479-82, 2002.

Schinkel, A. H. P-Glycoprotein, a gatekeeper in the blood-brain barrier. Adv Drug Deliv Rev 36: 179-194, 1999.

Schulze, C. Endothelium of the brain. In Bicknell, R. (Ed.) Endothelial cell culture. Great Britain, Cambridge University Press, 1996.

Selkoe, D. J. Alzheimer's disease: genes, proteins, and therapy. Physiol Rev 81: 741-66, 2001.

Sheiner, L. B. The population approach to pharmacokinetic data analysis: rationale and standard data analysis methods. Drug Metab Rev 15: 153-71, 1984.

Shen, D. D., Artru, A. A., Adkison, K. K. Principles and applicability of CSF sampling for the assessment of CNS drug delivery and pharmacodynamics. Adv Drug Deliv Rev 56: 1825-57, 2004.

Smith, Q. R. Quantitation of Blood-Brain Barrier Permeability. In Neuwelt, E. A. (Ed.) Implications of the Blood-Brain Barrier and Its Manipulation. New York, Plenum Medical Book Company, 1989.

Smith, Q. R. Brain perfusion systems for studies of drug uptake and metabolism in the central nervous system. In Borchardt, R., T. (Ed.) Models for assessing drug absorption and metabolism. New York, Plenum Press, 1996.

Smith, Q. R., Allen, D. D. In situ brain perfusion technique. Methods Mol Med 89: 209-18, 2003.

Smyth, E. M., Burke, A., FitzGerald, G. A. Lipid-Derived Autacoids: Eicosanoids and Platelet-Activating Factor. In Brunton, L. L., Lazo, J. S., Parker, K. L. (Eds.) Goodman, Gilman's The Pharmacological Basis of Therapeutics. 11th ed. New York, McGraw-Hill Companies, Inc, 2006.

Stewart, W. F., Kawas, C., Corrada, M., Metter, E. J. Risk of Alzheimer's disease and duration of NSAID use. Neurology 48: 626-32, 1997.

Stock, N., Munoz, B., Wrigley, J. D., Shearman, M. S., Beher, D., Peachey, J., Williamson, T. L., Bain, G., Chen, W., Jiang, X., St-Jacques, R., Prasit, P. The geminal dimethyl analogue of Flurbiprofen as a novel Abeta42 inhibitor and potential Alzheimer's disease modifying agent. Bioorg Med Chem Lett 16: 2219-23, 2006.

Sugiyama, D., Kusuhara, H., Taniguchi, H., Ishikawa, S., Nozaki, Y., Aburatani, H., Sugiyama, Y. Functional characterization of rat brain-specific organic anion transporter (Oatp14) at the blood-brain barrier: high affinity transporter for thyroxine. J Biol Chem 278: 43489-95, 2003.

Summerfield, S. G., Stevens, A. J., Cutler, L., del Carmen Osuna, M., Hammond, B., Tang, S. P., Hersey, A., Spalding, D. J., Jeffrey, P. Improving the in vitro prediction of in vivo central nervous system penetration: integrating permeability, P-glycoprotein efflux, and free fractions in blood and brain. J Pharmacol Exp Ther 316: 1282-90, 2006.

Summerfield, S. G., Read, K., Begley, D. J., Obradovic, T., Hidalgo, I. J., Coggon, S., Lewis, A. V., Porter, R. A., Jeffrey, P. Central Nervous System Drug Disposition: The Relationship between in Situ Brain Permeability and Brain Free Fraction. J Pharmacol Exp Ther 322: 205-13, 2007.

Szekely, C. A., Thorne, J. E., Zandi, P. P., Ek, M., Messias, E., Breitner, J. C., Goodman, S. N. Nonsteroidal anti-inflammatory drugs for the prevention of Alzheimer's disease: a systematic review. Neuroepidemiology 23: 159-69, 2004.

Takada, Y., Vistica, D. T., Greig, N. H., Purdon, D., Rapoport, S. I., Smith, Q. R. Rapid high-affinity transport of a chemotherapeutic amino acid across the blood-brain barrier. Cancer Res 52: 2191-6, 1992.

Takasato, Y., Rapoport, S. I., Smith, Q. R. An in situ brain perfusion technique to study cerebrovascular transport in the rat. Am J Physiol 247: H484-93, 1984.

Tamai, I., Tsuji, A. Transporter-mediated permeation of drugs across the blood-brain barrier. J Pharm Sci 89: 1371-88, 2000.

Tanaka, H., Mizojiri, K. Drug-protein binding and blood-brain barrier permeability. J Pharmacol Exp Ther 288: 912-8, 1999.

Terasaki, T., Ohtsuki, S., Hori, S., Takanaga, H., Nakashima, E., Hosoya, K. New approaches to in vitro models of blood-brain barrier drug transport. Drug Discov Today, 8: 944-54, 2003.

Townsend, K. P., Pratico, D. Novel therapeutic opportunities for Alzheimer's disease: focus on nonsteroidal anti-inflammatory drugs. Faseb J 19: 1592-601, 2005.

Triguero, D., Buciak, J., Pardridge, W. M. Capillary depletion method for quantification of blood-brain barrier transport of circulating peptides and plasma proteins. J Neurochem 54: 1882-8, 1990.

Tsuji, A. Specific Mechanisms for Transporting Drugs Into Brain. In Begley, D. J., Bradbury, M. W., Kreuter, J. (Eds.) The Blood-Brain Barrier and Drug Delivery to the CNS. New York, Marcel Dekker, Inc, 2000.

Tsuji, A., Saheki, A., Tamai, I., Terasaki, T. Transport mechanism of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors at the blood-brain barrier. J Pharmacol Exp Ther 267: 1085-90, 1993.

Upton, R. A., Williams, R. L., Guentert, T. W., Buskin, J. N., Riegelman, S. Ketoprofen pharmacokinetics and bioavailability based on an improved sensitive and specific assay. Eur J Clin Pharmacol 20: 127-33, 1981.

Van Amsterdam, C., Lemaire, M. Pharmacokinetic profile of SDZ EAA 494 in blood, brain and CSF using microdialysis. Eur J Pharm Sci 5: 109-16, 1997.

Vane, J. R. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nat New Biol 231: 232-5, 1971.

Verbeeck, R. K. Pharmacokinetic drug interactions with nonsteroidal anti-inflammatory drugs. Clin Pharmacokinet 19: 44-66, 1990.

Vlad, S. C., Miller, D. R., Kowall, N. W., Felson, D. T. Protective effects of NSAIDs on the development of Alzheimer's disease. Neurology 70: 1672-7, 2008.

Wang, Q., Yang, H., Miller, D. W., Elmquist, W. F. Effect of the P-glycoprotein inhibitor, cyclosporin A, on the distribution of rhodamine-123 to the brain: an in vivo microdialysis study in freely moving rats. Biochem Biophys Res Comm 211: 719-26, 1995.

Wijnholds, J., de Lange, E. C., Scheffer, G. L., van den Berg, D. J., Mol, C. A., van der Valk, M., Schinkel, A. H., Scheper, R. J., Breimer, D. D., Borst, P. Multidrug resistance protein 1 protects the choroid plexus epithelium and contributes to the blood-cerebrospinal fluid barrier. J Clin Invest 105: 279-85, 2000.

Wolburg, H., Lippoldt, A. Tight junctions of the blood-brain barrier: development, composition and regulation. Vascul Pharmacol 38: 323-37, 2002.

Xie, R., Hammarlund-Udenaes, M., de Boer, A. G., de Lange, E. C. M. The role of P-glycoprotein in blood-brain barrier transport of morphine: transcortical microdialysis in mdr1a(-/-) and mdr1a (+/+) mice. Br J Pharmacol 128: 563-8, 1999.

Yamazaki, M., Fukuoka, H., Nagata, O., Kato, H., Ito, Y., Terasaki, T., Tsuji, A. Transport mechanism of an H1-antagonist at the blood-brain barrier: transport mechanism of mepyramine using the carotid injection technique. Biol Pharm Bull 17: 676-9, 1994.

Yang, Z., Brundage, R. C., Barbhaiya, R. H., Sawchuk, R. J. Microdialysis studies of the distribution of stavudine into the central nervous system in the freely-moving rat. Pharm Res 14: 865-72, 1997.

Yan, R., Taylor, E. M. Neotrofin is transported out of brain by a saturable mechanism: possible involvement of multidrug resistance and monocarboxylic acid transporters. Drug Metab Dispos 30: 513-8, 2002.

Zandi, P. P., Breitner, J. C. Do NSAIDs prevent Alzheimer's disease? And, if so, why? The epidemiological evidence. Neurobiol Aging 22: 811-7, 2001.

Zhang, Y., Schuetz, J. D., Elmquist, W. F., Miller, D. W. Plasma membrane localization of multidrug resistance-associated protein homologs in brain capillary endothelial cells. J Pharmacol Exp Ther 311: 449-55, 2004.

3 AIMS OF THE STUDY

The rationale for the present thesis emerged from the need to understand better the CNS permeation and distribution properties of NSAIDs. The CNS availability of NSAIDs is of interest because NSAIDs may have clinically significant CNS actions related to central antinociception and prevention or treatment of neurodegenerative diseases such as AD. However, the CNS permeation of NSAIDs and the factors affecting the disposition of their CNS delivery are not fully understood. We hypothesized that the CNS permeation of traditional, widely used NSAIDs ibuprofen, indomethacin and ketoprofen is limited after systemic administration when the extent of the CNS permeation is evaluated based on the ratio between the brain or the CSF concentration and the plasma concentration. Therefore, the main aim of the present study was **to evaluate the CNS permeation of three NSAIDs, ibuprofen, ketoprofen and indomethacin.**

The main aim was divided into two parts; the first aim was to evaluate **the CNS permeation of ibuprofen and indomethacin in rats** by measuring the drug concentrations in brain tissue homogenate after intravenous infusion or after perfusion via the carotid artery. The second aim was to evaluate **the CSF permeation of ketoprofen and indomethacin in children** after intravenous injection.

Implementation of the experiments required **the refinement of the constant *in vivo* infusion method and an *in situ* brain perfusion method in rats**. The determination of ibuprofen, indomethacin and ketoprofen from biological samples, which included brain tissue homogenate, CSF, plasma and protein-free plasma, required **the development and the verification of the reliability of the sample pre-treatment as well as HPLC and GC-MS methods**.

4 CNS PERMEATION OF IBUPROFEN AND INDOMETHACIN IN RATS[*]

Abstract. Chronic use of NSAIDs may reduce the risk or delay the onset of Alzheimer's disease. To date, only limited information exists on the brain distribution of these drugs. The objective of this study was to determine the brain permeation of ibuprofen and indomethacin by using constant *in vivo* infusion in rats. In addition, the effect of an efflux protein inhibitor probenecid and plasma protein binding on brain permeation of indomethacin was evaluated using the *in vivo* method and an *in situ* brain perfusion technique. Drug levels in plasma and brain tissue were measured by RP-HPLC after the plasma and the brain samples were purified by protein precipitation and solid phase extraction, respectively. In addition, protein-free plasma concentrations of indomethacin were determined after ultrafiltration of the plasma samples. Results indicate that after a 6-hour drug infusion, the brain to plasma ratio of ibuprofen was 0.02, and the corresponding ratio for indomethacin was 0.01. Co-administration of probenecid increased total plasma, protein-free plasma and brain tissue concentrations, and the brain to plasma concentration ratio of indomethacin by 1.4-, 6.3-, 3.4, and 2.4-fold, respectively. The unidirectional brain uptake transfer constant (K_{in}) of indomethacin from buffer was $2.2 \pm 0.2 \times 10^{-3}$ ml/s/g (mean±SEM, n=3). Addition of 0.28 % or 2.8 % bovine serum albumin or probenecid into the perfusion fluid led to K_{in} values of $1.9 \pm 0.3 \times 10^{-3}$, $0.5 \pm 0.1 \times 10^{-3}$, and $3.7 \pm 0.3 \times 10^{-3}$ ml/s/g (mean±SEM, n=3-4), respectively. In conclusion, both ibuprofen and indomethacin permeated poorly into the rat brain after intravenous administration. Partly, this may be due to high plasma protein binding. However, a role for efflux systems at the blood-brain barrier cannot be ruled out because the brain permeation of indomethacin was increased by co-administration of the efflux inhibitor, probenecid.

4.1 Introduction

The CNS is protected by the blood-brain barrier (BBB) and the blood-CSF barrier (BCSFB). These barriers allow compounds to enter the CNS from the blood circulation either via transcellular diffusion or via selective carrier-mediated transport mechanisms. In theory, the low permeation of a drug into the CNS can be attributed to several factors e.g. to its inadequate lipophilicity, unsuitable peripheral pharmacokinetics (e.g. high degree of plasma protein binding), or interactions with efflux transport systems at the BBB/BCSFB. In the case of ibuprofen and indomethacin, all of the above mentioned factors may affect their CNS permeation. Firstly, both ibuprofen and indomethacin are weak acids and almost completely ionized at the pH of 7.4. It is generally believed that only unionized molecules, being lipophilic enough, pass readily through the BBB/BCSFB by transcellular diffusion and therefore, ionization of ibuprofen and indomethacin in the circulation can limit the ability of these drugs to permeate the CNS. Secondly, both ibuprofen and indomethacin are highly protein bound in the systemic circulation (Bannwarth *et al*. 1990, Summerfield *et al*. 2006) which may limit their permeation into the CNS. Thirdly, both ibuprofen and indomethacin have been shown to interact with several transport proteins, including multidrug resistance-associated proteins (MRPs) (Reid *et al*. 2003, Dallas *et al*. 2004), organic anion transporters (OATs) (Khamdang *et al*. 2002, Apiwattanakul *et al*. 1999), and organic anion transport polypeptides (OATPs) (Shitara *et al*. 2002). These transport protein families have been proposed to have a role as efflux transporters at the BBB/BCSFB (Zhang *et al*. 2004, Ohtsuki *et al*. 2002, Mori *et al*. 2004, Kusuhara and Sugiyama 2004, Kusuhara and Sugiyama 2005).

The CNS availability of ibuprofen and indomethacin is of interest because they may have clinically significant CNS actions in addition to their well-known peripheral anti-inflammatory and analgesic effects. For example, there is a growing amount of epidemiological, *in vitro* and *in vivo* evidence that certain NSAIDs including ibuprofen and indomethacin may have AD modifying properties (Szekely *et al*. 2004, Etminan *et al*. 2003, Townsend and Pratico 2005, Gasparini *et al*. 2004). Before they can be recommended for long-term use in the prevention of AD there are, however, several points which need to be clarified. Obviously, more clinical evidence about their effects in AD prevention is needed. In addition, since the site of action is expected to be in the CNS, the rate and extent of CNS permeation by the NSAIDs are a crucial issues and furthermore, one must determine whether their CNS availability is high enough to achieve their disease modifying effects at therapeutic dosages.

The aim of this study was to determine the brain permeation of ibuprofen and indomethacin after intravenous infusion by calculating the total brain to plasma ratio under steady-state conditions. In addition, the brain permeation of indomethacin was studied also with the *in situ* brain perfusion technique. The effect of an efflux inhibitor probenecid on brain permeation of indomethacin was evaluated both after intravenous administration, and after *in situ* brain

perfusion by the simultaneous administration of indomethacin and probenecid. In addition, the effect of plasma protein binding on the brain permeation of indomethacin was evaluated using the *in situ* brain perfusion method.

4.2 Experimental

4.2.1 Materials

Indomethacin, ibuprofen sodium salt, probenecid and bovine serum albumin (BSA, 98 %) were purchased from Sigma-Aldrich (Steinheim, Germany). Ketamine (Ketaminol) and xylazine (Narcoxyl) were purchased from Intervet International B.V. (Boxmeer, The Netherlands), sodium chloride solution (0.9 %) from Orion Pharma (Espoo, Finland), and heparin (Heparin Leo) from Leo Pharma (Ballerup, Denmark). Solvents were of HPLC grade and all other reagents were of analytical grade. The water used in the experiments was ultrapure.

4.2.2 Animals

Adult male Wistar rats weighing 250 (189-349) g (median with range) were purchased from the National Laboratory Animal Centre (Kuopio, Finland). Rats were housed in light- and temperature-controlled environment (12 hours light/dark cycle, 22 ± 1 °C). All experiments were carried out during the light phase. The rats had free access to tap water and food pellets. All procedures were reviewed and approved by the Animal Ethics Committee at the University of Kuopio.

4.2.3 *In situ* brain perfusion in rats

Rats were anesthetized with ketamine (90 mg/kg, i.p.) and xylazine (8 mg/kg, i.p.). A small incision was made in the neck on the right side of the midline and the right carotid artery system was exposed. The right external carotid artery was ligated, and the right common carotid artery was cannulated with PE-50 catheters filled with 100 IE/ml heparin. The pterygopalatine artery was left open. Blood flow from the systemic circulation of the rats was stopped by severing the cardiac ventricles, and the perfusion fluid was infused via the common carotid artery at a rate of 10 ml/min using Harvard PHD 22/2000 syringe pump (Harvard Apparatus Inc., Holliston, MA, USA).

The perfusion fluid contained 150 µg/ml indomethacin (target dose) in Krebs-bicarbonate buffer (2.4 mM NaH_2PO_4, 4.2 mM KCl, 24.0 mM $NaHCO_3$, 127.9 mM NaCl, 1.5 mM $CaCl_2$, 0.9 mM $MgCl_2$) and it also contained 1 % (v/v) dimethyl sulfoxide resulting from the use of a stock solution of indomethacin in dimethyl sulfoxide. The perfusion fluid was gassed with 95 % oxygen / 5 % carbon dioxide to pH 7.4, and 9 mM D-glucose was added to the solution. When the effect of probenecid and protein binding on indomethacin CNS permeation was

being studied, 240 µg/ml probenecid (target dose), 0.28 or 2.8 % (w/v) BSA were added to the solution. Prior to the perfusion, the perfusion fluid was filtered and heated to 37 °C. The perfusion procedure included a prewash for 30 s with indomethacin-free perfusion fluid, a perfusion with indomethacin for 30 s, and a wash period for 30 s with indomethacin-free perfusion fluid. When the effect of plasma protein binding was to be studied, BSA was perfused with indomethacin for 30 s. When the effect of probenecid was being investigated, also both washing periods included probenecid.

4.2.4 *In vivo* **infusion in rats**

Rats were anesthetized with ketamine (90 mg/kg, i.p.) and xylazine (8 mg/kg, i.p.). Small incisions were made in the neck to the right side of midline and in the region of the left groin. The right jugular and the left femoral veins were exposed aseptically (Waynforth and Flecknell 1994), and cannulated with PE-50 catheters filled with 100 IE/ml heparin in order to collect blood samples and to infuse drug solutions, respectively. The catheters were exteriorised through a small incision made in the back of the neck. The rats were allowed to recover until the following day.

Ibuprofen (sodium salt) and indomethacin solutions were prepared in 0.9 % NaCl and 20 mM phosphate buffered saline (pH 7.6), respectively, and filtered when necessary. Drug solutions were infused to conscious rats using a Univentor 864 syringe pump (Univentor Ltd., Zejtun, Malta). Ibuprofen (target dose 6 mg/kg/h) was infused for 2-6 hours, and indomethacin (target dose 7 mg/kg/h) was infused either in the absence of probenecid for 2-6 hours, or in the presence of probenecid for 6 hours. Probenecid was administered as an intravenous loading dose of 99 mg/kg followed by infusion of 26.6 mg/kg/h in order to obtain steady-state concentrations (Deguchi *et al*. 1997).

Blood samples were withdrawn before and during the infusion. The drawn blood volume was substituted by 0.9 % NaCl. The blood samples were promptly centrifuged after withdrawal, and the obtained plasma was immediately frozen on dry ice. The blood in the cerebral capillaries was taken into account either by using a calculatory approach (I) or by perfusion the brain (II) as follows: at the end of the infusion, the rat was anesthetized and the thorax was opened. A needle was inserted into the left ventricle of the heart, and the brains were perfused with 0.1 M phosphate buffered saline (pH 7.4) for 6 min in order to obtain blood-free brain. The time required was obtained from a separate set of studies (data not shown). The rats were decapitated, and the brains were removed from the cranium, and immediately frozen on dry ice.

4.2.5 Analytical methods

4.2.5.1 Sample preparation

Ibuprofen and indomethacin were isolated from the plasma samples by protein precipitation. Plasma (100 µl) was acidified with 50 µl of 1 M hydrochloric acid and vortexed for 5 minutes. Acetonitrile (1.0 ml) was added and the samples were vortexed for 2 minutes, after which time the samples were centrifuged for 5 minutes (5500 g). The supernatants were collected, and the samples were evaporated to dryness under a nitrogen stream at 40 °C. Prior to analysis, the samples were reconstituted in 1.0 ml of 45 % (v/v) acetonitrile in water, filtrated and analysed by RP-HPLC.

Ibuprofen and indomethacin were isolated from the brain samples by protein precipitation and solid phase extraction (SPE). A complete hemisphere was homogenized to obtain 3.0 ml of homogenate. Indomethacin or ibuprofen was added as an internal standard, and the samples were acidified with 300 µl of 2 M hydrochloric acid and vortexed for 5 minutes. Acetonitrile (4.0 ml) was added, and the samples were vortexed for 2 minutes, after which time the samples were centrifuged for 10 minutes (7500 g), and the supernatants were collected. The supernatants were diluted with 2.0 ml of ultrapure water and applied to the preconditioned and equilibrated C18 SPE-cartridges (Discovery DSC-18; Supelco, Bellefonte, PA, USA). The cartridges were first washed with 2.0 ml of 1 % (v/v) acetic acid solution to maintain ibuprofen and indomethacin in the unionized form, and then washed with 2.0 ml of water. The analytes were eluted with 3.0 ml of acetonitrile, and evaporated to dryness under a nitrogen stream at 40 °C. Prior to analysis, plasma and brain samples were reconstituted in 400 µl of 45 % (v/v) acetonitrile in water, filtrated and analysed by RP-HPLC.

The unbound fraction of indomethacin was determined from plasma samples obtained from the *in vivo* infusion studies and blank rat plasma spiked with indomethacin (160 µg/ml, n=3) and probenecid (390 µg/ml, n=3). In addition, the unbound fraction of indomethacin was determined from *in situ* perfusion fluid containing 0.28 or 2.8 % (w/v) of BSA. The plasma protein binding was determined by using the ultrafiltration method. Samples were applied to Centrifree® Micropartition Devices (Millipore Corporation, Bedford, MA, USA) and the protein-free fragment was obtained by centrifugation (1500 x g) for 15 minutes at 22 °C. The obtained ultrafiltrate was analysed by RP-HPLC.

4.2.5.2 High performance liquid chromatography procedure

The apparatus (Merck LaChrom isocratic RP-HPLC system, Hitachi, Tokyo, Japan) consisted of a D-6000A interface, L-6200A intelligent pump, L-4500 diode array detector, AS-2000 autosampler, L-7350 column oven, D-7000 chromatography data station software (version 3.1) and a Zorbax SB-C18 (4.6 X 150 mm, 5 µm) analytical column (Agilent Technologies Inc., Little Falls, Wilmington, DE, USA). The mobile phase was a mixture of

20 mM phosphate buffer solution (pH 2.5) and acetonitrile (46:54 and 55:45 v/v, for plasma and brain samples, respectively). Ten µl of plasma samples, 30 µl of brain samples and 50 µl of protein-free plasma samples, were injected to the apparatus and detected at a wavelength of 222 nm.

Ibuprofen: The calibration curves of the plasma and the brain methods were linear over a range of 0.2-50 µg/ml and 0.2-6 µg/ml, respectively. The precision of the plasma and the brain sample methods, were studied at a concentration level of the lower limit of quantification, and expressed as coefficients of variation (CV %); 4.6 % (n = 5) and 8.9 % (n = 4) for spiked samples containing 5 and 0.2 µg/ml of ibuprofen, respectively. The recovery was over 84 % with all studied methods.

Indomethacin: The plasma and brain assay procedures were linear over a range of 0.2-20 µg/ml and 0.2-6 µg/ml, respectively. The accuracy, precision (coefficient of variation, %) and recovery of the brain method were determined at concentration levels of 0.5 µg/ml and 2.5 µg/ml (n=3). Accuracy and recovery were 103-105 % and 77-79 %, respectively, and precision was 1.4 % at both concentrations. The precision and recovery of the plasma method were 2.1 % and 91 %, respectively, for spiked samples containing 4 µg/ml of indomethacin (n=6).

Ibuprofen and indomethacin concentrations in plasma were determined using external standards whereas the brain concentrations were assayed using an internal standard (ibuprofen for indomethacin and vice versa).

4.2.6 Calculations

The results are expressed as mean ± standard error of mean (SEM) of 3-4 rats. Ibuprofen and indomethacin concentrations in plasma and brain tissue after intravenous infusion were dose-normalized. Ibuprofen levels in brain parenchyma were calculated by correcting the measured brain sample concentrations for the vascular space component, which was set at 2 % (Morgan *et al*. 1992). Parenchymal brain concentrations of ibuprofen were calculated as follows:

$$C_i = C_m - V_p \times C_p \qquad\qquad (1)$$

where C_i is the brain concentration (extracellular + intracellular), C_m is the drug concentration in the brain sample, V_p is the fraction of brain tissue space occupied by plasma, and C_p is the drug concentration in plasma. In the case of indomethacin, C_i equals C_m because the cerebral capillaries were washed clean of blood prior to decapitation. The specific gravity of brain tissue was assumed to be 1.0 (Ohno *et al*. 1978).

The BBB transfer coefficient for unidirectional brain uptake (K_{in}) after *in situ* brain perfusion was calculated as follows (Smith and Allen 2003):

$$K_{in} = \frac{Q}{T \times C_{pf}} \qquad (2)$$

where K_{in} is the single point unidirectional transfer constant (ml/s/g brain), Q is the measured quantity of indomethacin in brain tissue (µg/g brain), T is the perfusion time (s), and C_{pf} is the perfusion fluid concentration of indomethacin (µg/ml).

The cerebrovascular permeability-surface area product (PA) after *in situ* brain perfusion for indomethacin from BSA-free perfusion fluid was calculated as follows (Smith and Allen 2003):

$$PA = -F \times \ln\left(1 - \frac{K_{in}}{F}\right) \qquad (3)$$

where PA is the cerebrovascular permeability-surface area product (ml/s/g brain) and F is the cerebral perfusion fluid flow (ml/s/g). F was experimentally determined in our laboratory from the brain uptake of diazepam and was 0.028 ml/s/g (1.68 ml/min/g) (Gynther *et al*. 2008).

Data were analyzed by SPSS software (version 14.0). Independent samples *t*-test was used to examine the differences between the plasma and brain tissue concentrations and brain to plasma concentration ratios after 6-hours of infusion with indomethacin in the absence or presence of probenecid. One-way analysis of variance with Dunnett t-test was used to examine differences in K_{in} values after *in situ* perfusion between the groups. A *p*-value of 0.05 was considered as the limit of statistical significance.

4.3 Results

4.3.1. Brain tissue and plasma concentrations after intravenous infusion

Ibuprofen and indomethacin concentrations in rat plasma and brain tissue were determined 2, 3, 4 and 6 hours after the start of continuous intravenous infusion. Total plasma concentrations ranged from 31 to 45 µg/ml for ibuprofen, and from 80 to 151 µg/ml for indomethacin. The brain tissue concentration ranged from 0.46 to 0.71 for ibuprofen, and from 0.45 to 1.7 µg/g for indomethacin (Table 4.1). Brain penetration, determined as total brain to total plasma concentration ration, was found to be extremely low for both ibuprofen and indomethacin. Both drugs had brain to total plasma concentration ratios of less than 0.02 (Table 4.1).

Table 4.1. Ibuprofen and indomethacin concentrations in rat plasma and brain tissue, and concentration ratios after constant infusion to freely moving rats (mean ± SEM, n=3). Drug concentrations are dose-normalized. IBU = ibuprofen target dose 6 mg/kg/h, IND = indomethacin target dose 7 mg/kg/h, IND + PROB = indomethacin target dose 7 mg/kg/h + probenecid target dose 99 mg/kg + 26.6 mg/kg/h.

Treatment	Sampling time	Plasma concentration (µg/ml)		Brain tissue concentration (µg/g)	Mean brain to plasma concentration ratio	
		Total	Protein-free[a]	Total	Total	Protein-free[a]
IBU	2 h	31 ± 9.2	0.47	0.46 ± 0.16	0.015	1.0
	3 h	35 ± 9.7	0.53	0.53 ± 0.21	0.015	1.0
	4 h	32 ± 2.5	0.48	0.69 ± 0.26	0.022	1.4
	6 h	45 ± 1.6	0.68	0.71 ± 0.26	0.016	1.0
IND	2 h	80 ± 7.4	0.21 ± 0.06[b]	0.45 ± 0.06	0.0057	2.0[b]
	3 h	87 ± 5.8	0.17[c]	0.67 ± 0.10	0.0077	2.9[c]
	4 h	131 ± 9.9	0.20 ± 0.05	1.2 ± 0.03	0.0095	7.4
	6 h	151 ± 11	0.39 ± 0.10	1.7 ± 0.24	0.011	4.9
IND + PROB	6 h	218 ± 9.4[d]	2.5 ± 0.12[d]	5.8 ± 0.7[d]	0.026[d]	2.6[b]

a = Calculated based on unbound fraction of 1.5% (Satterwhite and Boudinot 1991)
b = n=2
c = n=1
d = Values are significantly different from 6-hours infusion with indomethacin ($p<0.05$; independent samples t-test).

Our preliminary experiments with ibuprofen confirmed that the ibuprofen plasma steady-state was reached within 6 hours (Figure 4.1). The time needed for ibuprofen to reach steady-state in brain during intravenous infusion was not established by conducting preliminary experiments. Instead, it was decided to limit the infusion time to 6-hours based on the plasma concentration versus time profiles obtained in the preliminary experiments, and out of concern for the well-being of the animals. However, the results suggest that the brain ibuprofen concentrations were also at a steady-state after the 6 hour infusion (Figure 4.2). In contrast, it seemed that indomethacin steady-state was not achieved during the 6 hours of infusion since both the plasma and the brain concentrations increased with the passage of infusion time (Table 4.1).
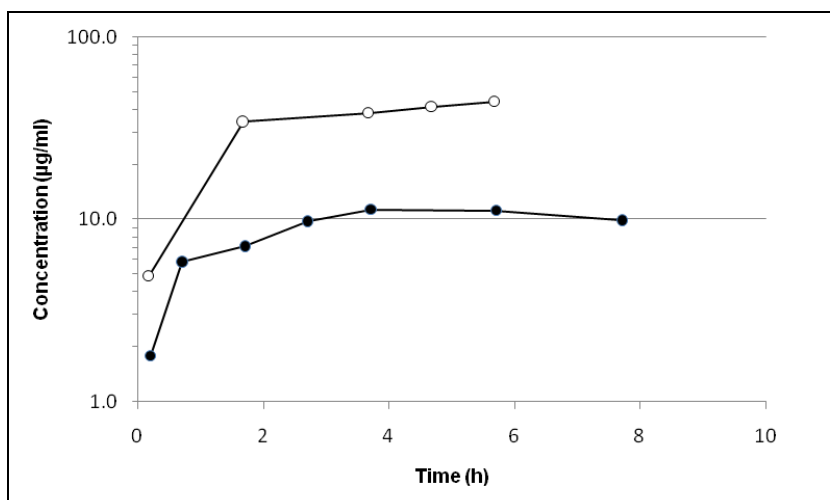
Figure 4.1. Individual plasma concentration versus time profiles of two rats during constant ibuprofen infusion at nominal dose of 6 mg/kg/h (○) and 1.2 mg/kg/h (●).
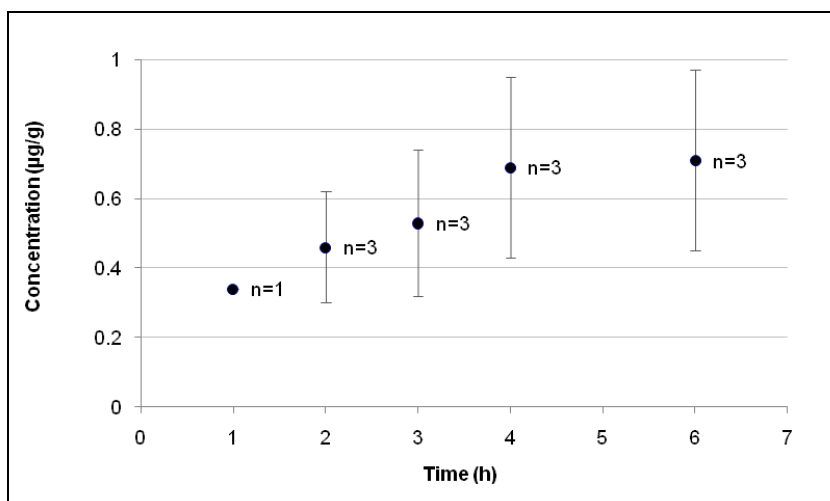
Figure 4.2. Ibuprofen concentrations in rat brain tissue during constant infusion of 6 mg/kg/h (mean ± SEM).

Simultaneous administration of indomethacin and probenecid for 6 hours increased the plasma and brain tissue concentrations of indomethacin by 1.4-fold and by 3.4-fold, respectively. Co-administration of probenecid increased the indomethacin brain to plasma concentration ratio by 2.4-fold (Table 4.1, Figure 4.3.).
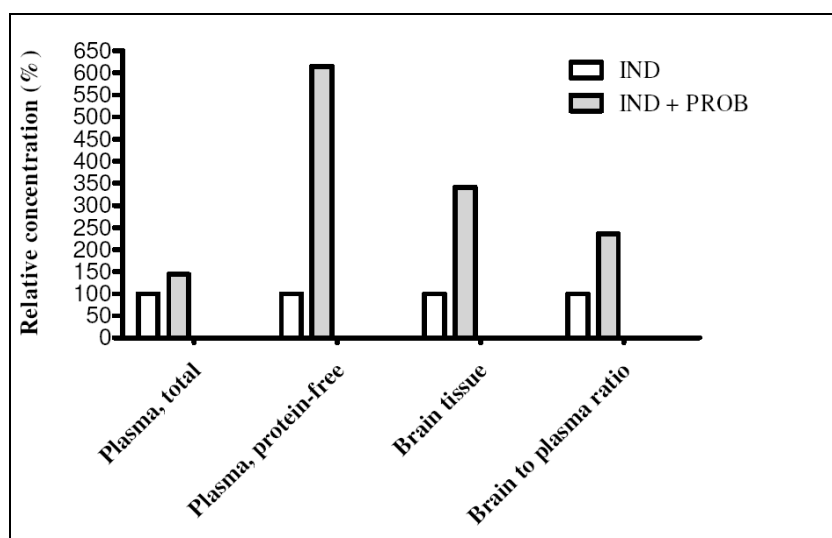
Figure 4.3. The effect of simultaneous probenecid administration on the plasma and brain tissue concentrations of indomethacin at 6 hours following continuous infusion of both compounds. IND = indomethacin target dose 7 mg/kg/h, IND + PROB = indomethacin target dose 7 mg/kg/h + probenecid target dose 99 mg/kg + 26.6 mg/kg/h. Data is presented as mean of 3 rats.

4.3.2. Plasma protein binding of indomethacin

The plasma protein binding of indomethacin was studied in the absence and presence of an efflux inhibitor probenecid. It was noted that indomethacin was extensively bound to plasma proteins but probenecid decreased the protein binding of indomethacin. The unbound fraction of indomethacin in the absence and presence of probenecid *in vitro* was 0.9 ± 0.1 % and 2.3 ± 0.3 % (mean±SD, n=3), respectively. The unbound fraction of indomethacin in plasma samples obtained after 6 hours of intravenous infusion was 0.2 % and 1.2 % in the absence and presence of probenecid, respectively (Table 4.1).

The unbound plasma concentration of indomethacin varied between 0.17 and 0.39 µg/ml after constant intravenous infusion (7 mg/kg/h) for 2-6 hours (Table 4.1). Co-administration of probenecid increased the unbound indomethacin concentration by 6.3-fold.

4.3.3. The effect of albumin and probenecid on brain permeation of indomethacin after *in situ* brain perfusion

Brain permeation of indomethacin was determined *in situ* after 30 s perfusion. In the absence of albumin or probenecid, the K_{in} and the PA value of indomethacin was 2.2 ± 0.2 x 10^{-3} ml/s/g brain and 2.3 x 10^{-3} ml/s/g brain, respectively. The addition of BSA into the perfusion fluid decreased the brain tissue concentration and K_{in} values of indomethacin

(Figure 4.3). The K_{in} value of indomethacin after perfusion with a perfusion fluid containing 0.28 % and 2.8 % (w/v) BSA was $1.9 \pm 0.3 \times 10^{-3}$ and $0.5 \pm 0.1 \times 10^{-3}$ ml/s/g brain, respectively. The unbound fractions of indomethacin in the perfusion fluid containing 0.28 % and 2.8 % (w/v) BSA were 40 % and 4 %, respectively.

When the brains were perfused with a solution containing both indomethacin and the efflux inhibitor, probenecid, the brain tissue concentration and the K_{in} value of indomethacin were higher than after indomethacin administration alone (Figure 4.4). The K_{in} value of indomethacin in the presence of probenecid was $3.7 \pm 0.3 \times 10^{-3}$ ml/s/g brain.
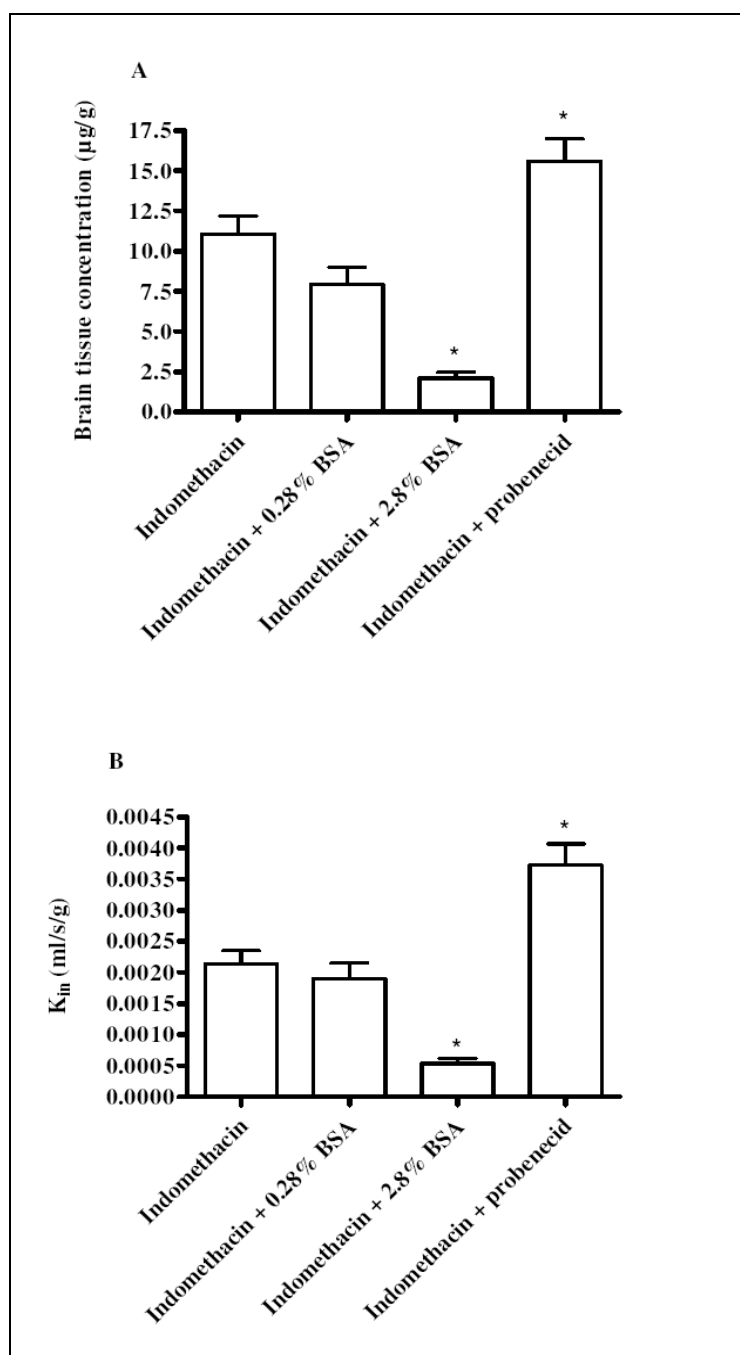
Figure 4.4. The effect of BSA or probenecid on the brain tissue concentration (A) and K_{in} value (B) of indomethacin after *in situ* brain perfusion (target indomethacin concentration in perfusion fluid 150 µg/ml). Values are expressed as mean±SEM. *, values are significantly different from indomethacin ($p<0.05$; one-way analysis of variance with Dunnett *t*-test).

4.4 Discussion and conclusions

Ibuprofen and indomethacin permeated poorly into the rat CNS after intravenous administration, as indicated by the low total brain to total plasma concentration ratio of 0.02 for ibuprofen and 0.011 for indomethacin. However, during the *in situ* brain perfusion, indomethacin entered the brain tissue quite readily with a PA value of 0.23×10^{-2} ml/s/g. A PA-value of 0.64×10^{-2} ml/s/g for indomethacin and 2.63×10^{-2} ml/s/g for ibuprofen has been reported in a previous study (Parepally *et al*. 2006). For comparison, a brain uptake of 2.8×10^{-2} ml/s/g has been determined for diazepam in our laboratory (Gynther *et al*. 2008). Diazepam permeates the brain rapidly and has even been used for the determination of the cerebral blood flow. Generally, if the compound has a PA-value that is much smaller than that of diazepam, it suggests that the brain uptake of the compound is being determined by factors other than the cerebral blood flow.

Ibuprofen levels in brain parenchyma were calculated by correcting the measured brain sample concentration for the cerebral vascular space component. The vascular space parameter is believed to have a value between 1 and 4 %, depending upon the method used (Preston *et al*. 1983). In the present study, a value of 2 % was adopted from the study by Morgan *et al*. (1992). The estimation of the vascular space component is critical for the quantification of drug concentration in brain tissue, especially when the concentration of the drug in plasma is high relative to that in brain parenchyma. In the case of ibuprofen, the calculations of brain parenhyma concentrations after 6-hours infusion using the vascular space value of 4 %, results in negative value in two rats out of three. This could suggest that ibuprofen does not enter the brain tissue at all, and the brain concentrations measured result only from ibuprofen present in the brain vasculature. However, it can also suggest that the contribution of the vascular component is overestimated, and the calculated brain parenchyma concentration is negative due to the relatively high plasma concentration. If one calculates the brain parenchyma concentration of ibuprofen using a vascular space value of 1 %, the brain concentrations of ibuprofen will be slighly higher, resulting in a brain to plasma ratio of 0.026 in comparison to 0.016 obtained in the present study. This suggests that inaccuracies in the estimation of the vascular space value do not alter the main conclusion that ibuprofen is extremely poorly delivered to the brain. When the brain parenchyma concentrations of indomethacin were calculated using the vascular correction, the concentrations were negative resulting from relatively high plama concentrations of indomethacin. That is why the indomethacin levels in brain parenchyma were determined from brain tissue that was perfused free from blood thus eliminating the vascular component. The perfusion was conducted immediately after the experiment by inserting a needle into the left ventricle of the heart followed by a 6-minute perfusion with phosphate buffer. After this perfusion procedure, there was no need to correct the measured brain tissue concentration for the cerebral vascular space component. Therefore, inaccuracy in the estimation of the vascular space component has no impact on the determined brain levels of indomethacin.

It is possible that the poor brain penetration of ibuprofen and indomethacin is due to efflux mechanisms expressed at the BBB/BCSFB. Several studies have reported that ibuprofen and indomethacin can interact with transporters suggested to have a role as efflux transporters at the BBB/BCSFB. These transporters include MRP1, 4 and 5 (Reid *et al*. 2003, Dallas *et al*. 2004), OAT1 and 3 (Khamdang *et al*. 2002, Apiwattanakul *et al*. 1999), and oatp1a1 and 1a4 (Shitara *et al*. 2002).

The effect of efflux transporters on the brain permeation of compounds can be evaluated *in vivo* in several ways. One of the most sophisticated approaches is to use transgenic animals which lack a specific efflux transporter. Currently, at least an mdr1a/1b knockout (i.e. P-gp deficient) mouse model and an mrp2-deficient rat model are available. Another approach includes the use of an efflux inhibitor. If the brain to plasma ratio of a compound is increased in the presence of the inhibitor, this can be interpreted as evidence for involvement of an efflux mechanism (Golden and Pollack 2003). In addition, the *in situ* brain perfusion technique can be used to evaluate the effect of efflux mechanisms by adding an efflux inhibitor to the perfusion fluid (Chikhale *et al*. 1995, Cisternino *et al*. 2003). In the present study, both *in vivo* and *in situ* methods were used to evaluate whether the brain permeation of indomethacin could be increased by co-administration of an efflux inhbitor. Probenecid, a rather non-specific inhibitor of several efflux proteins expressed at the BBB was used. It is known that probenecid interacts with many efflux proteins e.g. MRP, OAT, OCT and MCT transport systems (Deguchi *et al.* 1997, Dallas *et al*. 2004). Therefore, if one assumes that indomethacin is a substrate of a probenecid-sensitive efflux system, then the CNS permeation of indomethacin should be enhanced by concurrent administration of probenecid. According to the *in vivo* infusion studies, the brain permeation of indomethacin seems to be increased by co-administration with probenecid as the brain to plasma concentration ratio of indomethacin was increased by 2.4-fold when indomethacin was infused in conjunction with probenecid. In order to validate this assumption, an additional experiment where the steady-state conditions of indomethacin both in plasma and in brain tissue have been established would be required. Importantly, the steady-state levels of indomethacin would have to be established also in the presence of probenecid as it is likely that probenecid increases the time where the steady-state of indomethacin is reached. In addition to the *in vivo* infusion studies, the *in situ* experiments suggest that probenecid is able to increase the initial brain uptake of indomethacin from BSA-free perfusion fluid to some extent. This suggests that brain permeation of indomethacin may be influenced by a probenecid-sensitive efflux mechanism. The more precise identification of the efflux transporter possibly involved in the brain permeation of indomethacin cannot be established because probenecid, like many other commonly used efflux inhibitors, interacts with many different transport proteins. The effect of probenecid on the brain permeation of ibuprofen was also evaluated using the *in vivo* infusion method but our preliminary studies have suggested that probenecid has no effect on the brain permeation of ibuprofen (unpublished data).

In vivo infusion studies suggest that the brain permeation of indomethacin can be increased by co-administration of probenecid since the brain to plasma concentration ratio of indomethacin was increased by 2.4-fold when indomethacin was infused in conjunction with probenecid. In addition to the increase in the brain concentration of indomethacin, probenecid affected also the peripheral pharmacokinetics of indomethacin as reflected by the elevated indomethacin plasma levels. In particular, the 6.3-fold increase in the unbound concentration of indomethacin is noteworthy. Within the probenecid plasma concentration range used in the present study, probenecid is more than 60% bound to plasma proteins (Emanuelsson and Paalzow 1988). Therefore, probenecid may displace indomethacin from plasma proteins to some extent and thus increase the unbound plasma concentration of indomethacin. It is generally believed that only the unbound fraction of a drug is able to pass through the BBB/BCSFB. Therefore, it is possible that the increased brain to plasma concentration ratio of indomethacin after co-administration of probenecid is due to an increase in the unbound concentration of indomethacin in plasma.

The effect of plasma protein binding on brain permeation of indomethacin was further evaluated using the *in situ* brain perfusion method. The brain perfusion studies showed that in the absence of albumin, indomethacin entered the brain tissue with a K_{in} value of 2.2×10^{-3} ml/s/g. After brain perfusion with buffer containing 2.8 % (w/v) BSA, a K_{in} value of 0.5×10^{-3} ml/s/g was determined. The decrease in the brain uptake of indomethacin in the presence of albumin observed in the present and in a previous study (Parepally *et al*. 2006) is evidence that plasma protein binding plays a crucial role in brain permeation of indomethacin. In the study by Parepally *et al*., the role of plasma protein binding was also determined in the brain uptake of ibuprofen and another NSAID, flurbiprofen. With both compounds, a pronounced decrease in the brain uptake was observed when albumin was added to the perfusion fluid demonstrating that the brain permeation of these compounds is limited by plasma protein binding.

As discussed above, according to the *in situ* brain perfusion studies, the increase in unbound fraction enhances the brain permeation of indomethacin. The *in vivo* infusion experiments in the present study point in the same direction, i.e. the brain permeation of indomethacin was increased after there was an increase in the unbound fraction with the co-administration of probenecid. However, there are also opposite results to be found in the literature concerning the role of unbound drug concentration on the CNS permeation of NSAIDs. In the study of Matoga *et al*. (2002), ketoprofen was injected intraperitoneally to rats in either a carboxymethylcellulose solution or nanocapsule suspension. Despite the increase in the unbound fraction achieved with the latter formulation, there was no difference between the AUC_{CSF} / AUC_{plasma} ratios of ketoprofen after administration of these two formulations.

In conclusion, less than 1-2% of the total plasma concentrations of ibuprofen and indomethacin can be found in the brain parenchyma following intravenous infusion to rats, suggesting that ibuprofen and indomethacin permeates poorly into the rat brain following systemic administration. Simultaneous administration of the efflux protein inhibitor, probenecid, was able to increase the brain permeation of indomethacin. However, probenecid affected also the peripheral pharmacokinetics of indomethacin since both the total and unbound plasma concentrations were increased. The increase in the unbound fraction after co-administration of probenecid may account for the enhanced brain permeation of indomethacin after intravenous administration. This hypothesis is supported by the results from brain perfusion studies where it was observed that binding to BSA reduced the initial brain permeation of indomethacin. However, an effect on efflux systems at the BBB cannot be excluded as co-administration of probenecid slightly increased the initial brain uptake of indomethacin from protein-free perfusate. If one considers this result in terms of Alzheimer's disease prevention, then it does seem that the brain permeation of ibuprofen and indomethacin may not be large enough, because high plasma concentrations may be required in order to ensure adequate brain concentrations. However, from the adverse effect point of view, exposure to high doses of indomethacin would be most undesirable.

4.5 References

Apiwattanakul, N., Sekine, T., Chairoungdua, A., Kanai, Y., Nakajima, N., Sophasan, S., Endou, H. Transport properties of nonsteroidal anti-inflammatory drugs by organic anion transporter 1 expressed in Xenopus laevis oocytes. Mol Pharmacol, 55: 847-54, 1999.

Bannwarth, B., Netter, P., Lapicque, F., Pere, P., Thomas, P., Gaucher, A. Plasma and cerebrospinal fluid concentrations of indomethacin in humans. Relationship to analgesic activity. Eur J Clin Pharmacol, 38: 343-6, 1990.

Chikhale, E. G., Burton, P. S., Borchardt, R. T. The effect of verapamil on the transport of peptides across the blood-brain barrier in rats: kinetic evidence for an apically polarized efflux mechanism. J Pharmacol Exp Ther 273: 298-303, 1995.

Cisternino, S., Rousselle, C., Debray, M., Scherrmann, J. M. In vivo saturation of the transport of vinblastine and colchicine by P-glycoprotein at the rat blood-brain barrier. Pharm Res 20: 1607-11, 2003.

Dallas, S., Schlichter, L., Bendayan, R. Multidrug resistance protein (MRP) 4- and MRP 5-mediated efflux of 9-(2-phosphonylmethoxyethyl)adenine by microglia. J Pharmacol Exp Ther 309: 1221-9, 2004.

Deguchi, Y., Nozawa, K., Yamada, S., Yokoyama, Y., Kimura, R. Quantitative evaluation of brain distribution and blood-brain barrier efflux transport of probenecid in rats by microdialysis: possible involvement of the monocarboxylic acid transport system. J Pharmacol Exp Ther 280: 551-60, 1997.

Emanuelsson, B. M., Paalzow, L. K. Dose-dependent pharmacokinetics of probenecid in the rat. Biopharm Drug Dispos 9: 59-70, 1988.

Etminan, M., Gill, S., Samii, A. Effect of non-steroidal anti-inflammatory drugs on risk of Alzheimer's disease: systematic review and meta-analysis of observational studies. BMJ 327: 128-32, 2003.

Gasparini, L., Ongini, E., Wenk, G. Non-steroidal anti-inflammatory drugs (NSAIDs) in Alzheimer's disease: old and new mechanisms of action. J Neurochem 91: 521-36, 2004.

Golden, P. L., Pollack, G. M. Blood-brain barrier efflux transport. J Pharm Sci 92: 1739-53, 2003.

Gynther, M., Laine, K., Ropponen, J., Leppänen, J., Mannila, A., Nevalainen, T., Savolainen, J., Järvinen, T., Rautio, J. Large Neutral Amino Acid Transporter Enables Brain Drug Delivery via Prodrugs. J Med Chem 51: 932-6, 2008.

Khamdang, S., Takeda, M., Noshiro, R., Narikawa, S., Enomoto, A., Anzai, N., Piyachaturawat, P., Endou, H. Interactions of human organic anion transporters and human organic cation transporters with nonsteroidal anti-inflammatory drugs. J Pharmacol Exp Ther 303: 534-9, 2002.

Kusuhara, H., Sugiyama, Y. Efflux transport systems for organic anions and cations at the blood-CSF barrier. Adv Drug Deliv Rev 56: 1741-63, 2004.

Kusuhara, H., Sugiyama, Y. Active efflux across the blood-brain barrier: role of the solute carrier family. NeuroRx 2: 73-85, 2005.

Matoga, M., Pehourcq, F., Lagrange, F., Fawaz, F., Bannwarth, B. Influence of a polymeric formulation of ketoprofen on its diffusion into cerebrospinal fluid in rats. J Pharm Biomed Anal 27: 881-8, 2002.

Mori, S., Ohtsuki, S., Takanaga, H., Kikkawa, T., Kang, Y. S., Terasaki, T. Organic anion transporter 3 is involved in the brain-to-blood efflux transport of thiopurine nucleobase analogs. J Neurochem 90; 931-41, 2004.

Ohtsuki, S., Asaba, H., Takanaga, H., Deguchi, T., Hosoya, K., Otagiri, M., Terasaki, T. Role of blood-brain barrier organic anion transporter 3 (OAT3) in the efflux of indoxyl sulfate, a uremic toxin: its involvement in neurotransmitter metabolite clearance from the brain. J Neurochem 83: 57-66, 2002.

Parepally, J. M., Mandula, H., Smith, Q. R. Brain Uptake of Nonsteroidal Anti-Inflammatory Drugs: Ibuprofen, Flurbiprofen, and Indomethacin. Pharm Res 23: 873-81, 2006.

Reid, G., Wielinga, P., Zelcer, N., van der Heijden, I., Kuil, A., de Haas, M., Wijnholds, J., Borst, P. The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs. Proc Natl Acad Sci U S A 100: 9244-9, 2003.

Satterwhite, J. H., Boudinot, F. D. Effects of age and dose on the pharmacokinetics of ibuprofen in the rat. Drug Metab Dispos 19: 61-67, 1991.

Shitara, Y., Sugiyama, D., Kusuhara, H., Kato, Y., Abe, T., Meier, P. J., Itoh, T., Sugiyama, Y. Comparative inhibitory effects of different compounds on rat oatpl (slc21a1)- and Oatp2 (Slc21a5)-mediated transport. Pharm Res 19: 147-53, 2002.

Smith, Q. R., Allen, D. D. In situ brain perfusion technique. Methods Mol Med 89: 209-18, 2003.

Summerfield, S. G., Stevens, A. J., Cutler, L., del Carmen Osuna, M., Hammond, B., Tang, S. P., Hersey, A., Spalding, D. J., Jeffrey, P. Improving the in vitro prediction of in vivo central nervous system penetration: integrating permeability, P-glycoprotein efflux, and free fractions in blood and brain. J Pharmacol Exp Ther 316: 1282-90, 2006.

Szekely, C. A., Thorne, J. E., Zandi, P. P., Ek, M., Messias, E., Breitner, J. C., Goodman, S. N. Nonsteroidal anti-inflammatory drugs for the prevention of Alzheimer's disease: a systematic review. Neuroepidemiology 23: 159-69, 2004.

Townsend, K. P., Pratico, D. Novel therapeutic opportunities for Alzheimer's disease: focus on nonsteroidal anti-inflammatory drugs. Faseb J 19: 1592-601, 2005.

Waynforth, H. B., Flecknell, P. A. Experimental and surgical technique in the rat, London, Academic press limited, 1994.

76

Zhang, Y., Schuetz, J. D., Elmquist, W. F., Miller, D. W. Plasma membrane localization of multidrug resistance-associated protein homologs in brain capillary endothelial cells. J Pharmacol Exp Ther 311: 449-55, 2004.

5 CSF PERMEATION OF KETOPROFEN AND INDOMETHACIN IN CHILDREN[*]

Abstract. The objective of this study was to evaluate the cerebrospinal fluid (CSF) permeation of ketoprofen and indomethacin in healthy children. The participants (age 4-144 months) received either ketoprofen (1 mg/kg) or indomethacin (0.35 mg/kg) intravenously prior to surgery under spinal anaesthesia. A single CSF and plasma sample from each individual was collected 7-67 minutes after the ketoprofen infusion, and 14-225 minutes after the indomethacin infusion. Drug concentrations were determined from the CSF, plasma and protein-free plasma using GC-MS. Total plasma, protein-free plasma and CSF concentrations of ketoprofen varied between 1700-9500 ng/ml (median 5800 ng/ml), 6.2-97 ng/ml (median 35 ng/ml) and 1.4-24 ng/ml (median 6.6 ng/ml), respectively. Total plasma, protein-free plasma and CSF concentrations of indomethacin was in the range 90-2200 ng/ml (median 780 ng/ml), 0.3-0.8 ng/ml (median 0.5 ng/ml) and 0.2-5.0 ng/ml (median 1.4 ng/ml), respectively. The CSF to plasma concentration ratio of both drugs remained below 0.01. In conclusion, both ketoprofen and indomethacin did permeate into the CSF of children after intravenous administration which may lead to both desired and adverse CNS effects of these drugs.

5.1 Introduction

Ketoprofen and indomethacin are traditional non-steroidal anti-inflammatory drugs (NSAIDs). They have analgesic, anti-inflammatory and antipyretic properties, which are thought to result mainly from peripheral inhibition of the cyclooxygenase (COX) enzyme at the site of tissue damage. In children, they are used for the treatment of inflammation and pain resulting from rheumatic and orthopaedic diseases and surgery (Maunuksela *et al*. 1988). In addition, indomethacin is the most commonly used pharmacological agent for the closure of the patent ductus arteriosus in preterm infants (Van Overmeire and Chemtob 2005).

In addition to peripheral sites of action, ketoprofen and indomethacin act also at the central level (Ossipov *et al*. 2000, Malmberg and Yaksh 1992, Daher and Tonussi 2003), prostaglandin E_2 receptors being the key signalling elements where the spinal inflammatory hyperalgesia is inhibited (Reinold *et al*. 2005). In order to have central effects, a drug has to pass the blood-brain barrier and/or the blood-cerebrospinal fluid (CSF) barrier, which separate circulating blood from the brain and the CSF, respectively. These barriers efficiently control the exchange of substances between blood and the central nervous system (CNS). In principle, a drug molecule has to be small and lipophilic enough or a substrate for a transport protein located at the blood-brain/CSF barrier to be able to pass into the CNS. The CNS distribution of the NSAIDs (including ketoprofen and indomethacin) is largely unknown. Since there are CNS adverse effects such as dizziness, vertigo, headache, agitation and even psychosis and depression (Clunie *et al.* 2003, Tharumaratnam *et al*. 2000, Hoppmann *et al*. 1991) often associated with the use of indomethacin, it has been suggested that indomethacin can penetrate into the CNS quite readily. However, determination of drug concentration in the CNS is required to validate this assumption.

There are few studies describing the CSF permeation of ketoprofen and indomethacin in adults. Netter *et al*. (1985) have shown that ketoprofen is able to pass the blood-CSF barrier in adults, since it was detected in the CSF between 15 minutes and 13 hours after intramuscular administration. The CSF concentrations of indomethacin have been studied in adults by Dittrich *et al*. (1984) and Bannwarth *et al*. (1990). Though Dittrich and co-workers could not detect indomethacin in the CSF, probably due to their insensitive analytical method, Bannwarth and co-workers demonstrated that indomethacin is able to enter the CSF. At present, there has been only one attempt to evaluate the CNS pharmacokinetics of ketoprofen in children (Kokki *et al*. 2002). In that study, the insensitivity of the analytical method permitted the detection of ketoprofen in only one CSF sample. The patient's ketoprofen concentrations in the CSF and plasma after 30 minutes of oral administration were 0.06 and 7.4 µg/ml, respectively. This led to only limited information on CNS pharmacokinetics of ketoprofen in children, but did suggest that ketoprofen may be able to enter the CSF of children. We are unaware of any reports concerning the CSF permeation of indomethacin in

children. Consequently, more information is needed to clarify the CSF distribution of ketoprofen and indomethacin in children.

In the present study, the CSF distribution of ketoprofen and indomethacin was evaluated in children, aged 4-144 months. Children were given ketoprofen or indomethacin intravenously prior to surgery with spinal anaesthesia. Simultaneous venous blood and CSF samples were collected once from each patient. Samples were collected 7-67 minutes after ketoprofen administration and 14-225 minutes after indomethacin administration. Drug concentrations were determined in CSF, plasma and protein free plasma samples, and the concentration ratio between CSF and plasma was calculated in order to evaluate the CSF permeation.

5.2 Experimental

5.2.1 Materials

Ketoprofen (Orudis 50 mg/ml, Aventis Pharma S.A., Alcorcon, Spain) and indomethacin (Confortid 50 mg/ml: Alpharma, Copenhagen, Denmark) were used in the clinical studies. Ketoprofen, indomethacin, flurbiprofen and diclofenac used in the analytical studies were purchased from Sigma-Aldrich (Steinheim, Germany). All reagents used in the studies were of analytical grade, and the water was ultrapure.

5.2.2 Clinical protocol

The studies were open and prospective and they were conducted in the Kuopio University Hospital, Finland. The protocols were approved by the Research Ethics Committee of the Hospital District of Northern Savo, and the Finnish National Agency for Medicines was notified. The trials were conducted in accordance with the Declaration of Helsinki. The parents, and children if old enough, were informed and the parents gave written informed consent and children provided assent.

The enrolled children were scheduled for lower abdominal, genito-urinary, orthopaedic surgery on the lower extremities and gastrointestinal surgery, all to be performed under spinal anaesthesia as day-case surgery. The exclusion criteria were contraindications for the drugs to be investigated or for spinal anaesthesia in their medical and surgical history and in a physical examination of vital signs.

The children received ketoprofen and indomethacin as a single intravenous infusion which lasted for 5-10 minutes. The blood and CSF samples were collected at pre-selected time points after the end of the drug infusion. One ml of CSF was collected at the induction of spinal analgesia. At the same time, a venous blood sample of 3 ml was collected into a heparinised tube via an indwelling catheter inserted into the contralateral forearm vein

compared to the vein used for the infusion. Plasma was obtained by centrifugation and the plasma and the CSF samples were protected from light and stored at -85 °C until analysis.

After the surgery, the children were transferred to the post-anaesthesia care unit for monitoring of vital signs, pain and all adverse effects (not classified as to whether considered as drug-related or not).

Ketoprofen: 24 children were asked to participate but the parents of two children refused to provide informed consent, and one child was withdrawn because insertion of an intravenous line was not successful. Hence the study population was 21 children. The children were sedated before spinal anaesthesia with thiopental sodium.

The children received ketoprofen at a dose of 1 mg/kg in 20 ml of normal saline. The blood and CSF samples were collected at 7 min to 67 min (median 23 min) after the end of the ketoprofen infusion.

Indomethacin: 32 children were asked to participate; the parents of one child refused to provide informed consent, hence leaving a group of 31 children for the study population. The children were premedicated with buccal midazolam (0.375 mg/kg up to 7.5 mg) and ketamine (1.25 mg/kg up to 25 mg) 15 - 30 min before anaesthesia. All children were sedated before spinal anaesthesia (n=26) or combined spinal-epidural anaesthesia (n=5).

The children received indomethacin at a dose of 0.35 mg/kg. The blood and CSF samples were collected at 14 min to 3 hours 45 min (median 58 min) after the end of the indomethacin infusion.

5.2.3 Analytical methods

5.2.3.1 Sample preparation

Ketoprofen and indomethacin concentrations in CSF, plasma and protein-free plasma were determined by GC-MS. Protein-free plasma was obtained by the ultrafiltration method; plasma was placed into Centrifree® ultrafiltration devices (Millipore, Bedford, MA, USA) and centrifuged for 15 minutes at 1500 *g* at 23 °C. A SPE method was developed for sample pre-treatment. A method previously described by Leis *et al*. (1996) with modifications was used for sample derivatisation.

Ketoprofen: CSF (250 µl), protein-free plasma (75-100 µl), or plasma (100 µl) sample, containing the internal standard flurbiprofen, was acidified with 20 µl of 1 M hydrochloric acid, and applied into C-18 SPE-cartridges (Discovery®, DSC-18, 1ml/100mg, Supelco, Bellefonte, PA, USA). The cartridges were washed with 20 % (v/v) methanol (1.0 ml), and

ketoprofen and the internal standard were eluted with ethyl acetate (1.0 ml). After evaporation, 100 µl of pentafluorobenzylbromide (3.5 % v/v, in acetonitrile) and 20 µl of di-isopropylethylamine were added to the samples. After one hour, the samples were extracted with water and toluene. In the case of the CSF and total plasma samples, the ketoprofen concentration was analysed from the toluene phase, whereas in the case of protein-free plasma samples, the samples were further derivatised. The toluene phase was evaporated to dryness and 50 µl of hydroxylamide hydrochloride (3 %, in pyridine) were added. After two hours at 75 °C, the samples were extracted with water and hexane. The hexane phase was collected and evaporated, and then 50 µl of pyridine and 100 µl of bis(trimethylsilyl)trifluoracetamide were added to the residue. After 20 min in 75 °C, samples were evaporated, dissolved in toluene and analysed.

Indomethacin: CSF (250 µl), protein-free plasma (120-200 µl), or plasma (50-100 µl) sample, containing the internal standard diclofenac, was acidified and applied into C-18 SPE-cartridges (Discovery®, DSC-18, 1ml/100mg, Supelco, Bellefonte, PA, USA). Cartridges were washed with 20 % (v/v) methanol (1.0 ml), and indomethacin and the internal standard were eluted with ethyl acetate (2.0 ml). After evaporation, 200 µl of pentafluorobenzylbromide (3.5 % v/v, in acetonitrile) and 50 µl of di-isopropylethylamine were added to samples. After two hours, the samples were extracted with water and toluene, and the toluene phase was analysed.

5.2.3.2 Gas-chromatography mass-spectrometry procedure

GC-MS operating in the negative chemical ionisation was used for the determination of ketoprofen and indomethacin concentrations in CSF and plasma samples. The determinations were accomplished by the Agilent GC-NCI-MS system (6890 N gas chromatograph, 7683 autosampler and 5973 N mass detector, Agilent Technologies, Palo Alto, CA, USA). Data were processed by the Agilent Enhanced Chemstation software (version c.00.01.08). A cross-linked 5 % phenyl methyl siloxane capillary column (HP-5MS; 30 m x 0.25 mm x 0.25 µm, Agilent Technologies) was used with helium as the carrier gas (1.0-1.5 ml/min). Methane was used as the reagent gas.

Ketoprofen: The sample (1 µl for CSF and total plasma samples and 3 µl for protein-free plasma samples) was injected into the system in the pulsed splitless mode. The temperature program was as follows: 1 min at 90 °C, followed by an increase of 20 °C/min to 280 °C. Final temperature was held for 3.5 min. Selected ion monitoring at m/z 253 and 340 for ketoprofen pentafluorobenzylbromide ester and pentafluorobenzylbromide ester hydroxylamine-trimethylsilyl derivative, respectively, and 243 for flurbiprofen was used to quantify the compounds.

Indomethacin: The sample (1 µl) was injected in the splitless mode. The temperature program was as follows: 1 min at 180 °C, followed by an increase of 20 °C/min to 300 °C. Final temperature was held for 7 min. Selected ion monitoring at *m/z* 356 for indomethacin, and 294 for diclofenac was used to quantify the compounds.

5.2.4 Statistics

Drug concentrations in the CSF and plasma (total and protein-free plasma) are presented for each individual along with median supplemented with range or average with standard deviation (SD), as appropriate. The impact of administration time and the child's age on drug CSF concentration was tested using linear regression analysis. Mann-Whitney *U*-test was used to test if the indomethacin CSF concentration, child's age or administration time were associated with the observed CNS adverse effects. A p-value of 0.05 was considered as the limit of statistical significance. Data was analysed by SPSS software (version 11.5).

5.3 Results

5.3.1 Analytical methods

The methods for the determination of ketoprofen concentration from human CSF and plasma (total and protein-free concentration) were partially validated with regard to linearity, accuracy and precision. The range of the method was 0.4-40, 1000-30000 and 1-100 ng/ml for CSF, total plasma and protein-free plasma samples, respectively.

The accuracy and intra-day precision of the methods for the determination of indomethacin concentration from human CSF and plasma (total and protein-free concentration) and recovery from human plasma were studied in the following concentrations: 0.2, 3/4, and 16/20 ng/ml for CSF/plasma ultrafiltrate method, and 90 and 150 ng/ml for plasma method. The accuracy and recovery of the methods were in the ranges 98-122 % and 85-87 %, respectively. The intra-day precision (% RSD, n=3) of the methods was 34 %, 15 %, 6 %, 7 % and 3 % at concentrations of 0.2, 3/4, 16/20, 90 and 150 ng/ml, respectively. CSF and plasma ultrafiltrate methods were linear over the concentrations of 0.2-20 ng/ml and the plasma method over the concentrations of 90-3000 ng/ml.

5.3.2 CSF and plasma concentrations

The drug concentrations in CSF, plasma and protein-free plasma analysed from each patient are presented in Tables 5.1 and 5.2. Ketoprofen was detected in each CSF sample, whereas in two indomethacin samples, the drug concentration in the CSF was below the limit of quantitation. In addition, two indomethacin CSF samples were excluded from analysis due to blood contamination.

The ketoprofen concentration in the CSF ranged from 1.4 to 24 ng/ml (median 6.6 ng/ml) after the dose of 1 mg/kg. The indomethacin concentrations in the CSF were in the range from 0.2 to 5.0 ng/ml (median 1.4 ng/ml) after the dose of 0.35 mg/kg. The impact of time to CSF concentrations was tested using linear regression analysis. CSF concentrations of ketoprofen increased statistically significantly with increasing time ($p = 0.026$). There was no correlation between indomethacin CSF concentration and the time from indomethacin administration (Figure 5.1).
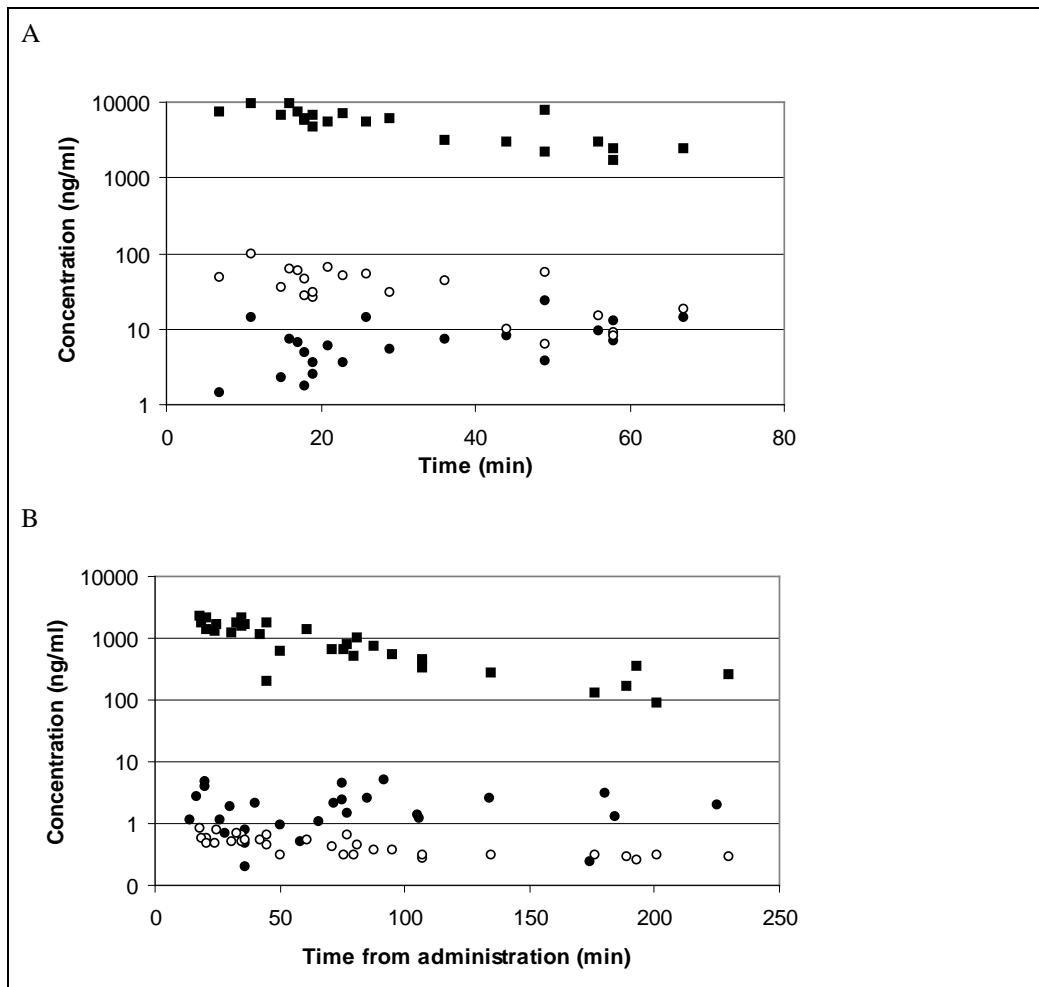
Figure 5.1. Total plasma (■), protein-free plasma (○) and CSF (●) concentrations of ketoprofen (A) and indomethacin (B) after intravenous administration in all patients.

Table 5.1. Ketoprofen concentrations in the CSF and plasma (total and protein-free plasma concentration). The study population included 13 males and 8 females, aged 13-94 (43) months, weighing 8-33 (14) kg (range with median).

Sampling time (min)	Concentration (ng/ml)			Age (mo)
	CSF	Plasma, protein-free	Plasma, total	
7	1.4	48	7514	18
11	14.0	97	9459	55
15	2.3	35	6717	43
16	7.3	63	9422	13
17	6.6	58	7436	74
18	4.7	46	5866	25
18	1.8	28	5757	79
19	3.5	26	4752	86
19	2.5	30	6643	78
21	6.0	66	5388	39
23	3.6	51	6900	25
26	14.4	52	5406	28
29	5.3	31	6111	94
36	7.5	44	3100	13
44	7.9	10	2959	91
49	24.0	56	7745	15
49	3.8	6.2	2125	73
56	9.4	15	2954	25
58	12.5	8.8	2411	41
58	7.0	7.9	1654	64
67	13.8	18	2461	65
Mean	7.6	38	5370	50
SD	5.5	23	2378	28
Range	1.4-24	6.2-97	1654-9459	13-94
Median	6.6	35	5757	43

Table 5.2. Indomethacin concentrations in the CSF and plasma (total and protein-free plasma concentration), and observed CNS adverse effects for each individual.

Sampling time (min)	Concentration (ng/ml)			Gender	Age (mo)	Height (cm)	Weight (kg)
	CSF	Plasma, protein-free	Plasma, total				
14	bq	0.8	2200	M	121	135	33
14	1.2	0.6	2200	M [a]	14	76	11
15	c	0.6	1700	M [a]	20	84	11
17	2.6	0.5	1400	F	82	115	18
19	bq	0.5	1300	M	115	120	28
20	3.8	0.8	1700	M	19	82	11
20	4.7	0.6	2200	M	6	66	7
26	1.2	0.5	1200	M	22	80	12
28	0.7	0.7	1800	M	51	101	17
30	1.9	0.5	1600	F [a]	31	89	12
36	0.8	0.4	200	M [a]	19	82	11
36	0.2	0.5	1600	M	64	120	19
36	0.5	0.5	1100	F	107	140	28
40	2.1	0.7	1700	M [a]	70	120	22
50	1.0	0.3	620	M	45	98	15
58	0.5	0.6	1300	M	107	124	22
66	1.0	0.4	660	F	37	89	13
72	2.1	0.5	1000	F	18	84	11
75	4.4	0.3	650	M	25	89	12
75	2.4	0.3	510	M	31	91	10
77	1.4	0.6	780	M	72	119	21
85	2.6	0.4	750	F	25	89	12
92	5.0	0.4	520	M	16	75	10
105	1.4	0.3	330	F	116	128	23
106	1.2	0.3	440	M	69	110	20
134	2.5	0.3	260	M	117	152	45
174	0.2	0.3	130	M	96	123	20

Table 5.2. continued…

Sampling time (min)	Concentration (ng/ml)			Gender	Age (mo)	Height (cm)	Weight (kg)
	CSF	Plasma, protein-free	Plasma, total				
180	3.0	0.3	350	M	4	63	7
184	1.3	0.3	160	F	15	82	13
200	c	0.3	90	M	94	123	24
225	2.0	0.3	250	M	144	165	48
Mean	1.9	0.5	990	-	57	104	18
SD	1.3	0.2	670	-	42	26	10
Range	≤ 5.0	0.3-0.8	90-2200	-	4-144	63-165	7-48
Median	1.4	0.5	780	-	45	98	15

a. CNS adverse effect (agitation)

bq = below quantitation limit; c = sample contaminated; F = female; M = male

The ketoprofen plasma concentrations ranged from 1700 to 9500 ng/ml (median 5800 ng/ml). Total plasma concentrations of indomethacin ranged from 90 to 2200 ng/ml (median 780 ng/ml). The CSF to plasma concentration ratio of both ketoprofen and indomethacin remained less than 0.01 at all times (Figure 5.2). The concentration ratio of ketoprofen ranged from 0.0002 to 0.006 (median 0.001) and the concentration ratio of indomethacin from 0.0001 to 0.001 (median 0.002).
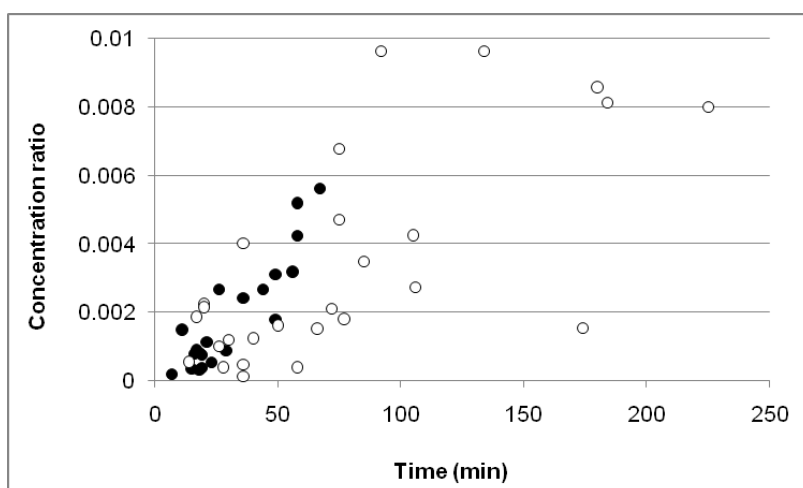
Figure 5.2. CSF to total plasma concentration ratio of ketoprofen (●) and indomethacin (○) from each patient.
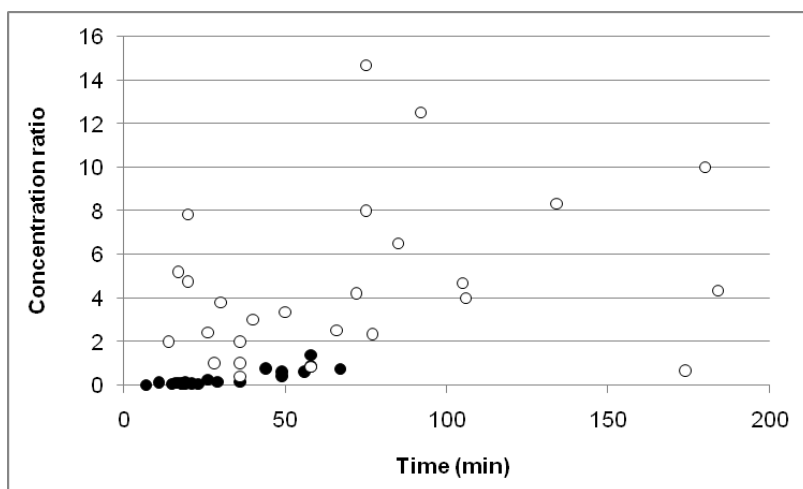
Figure 5.3. CSF to protein-free plasma concentration ratio of ketoprofen (●) and indomethacin (○) from each patient.

Both ketoprofen and indomethacin were extensively bound to plasma proteins. The unbound fraction of ketoprofen ranged from 0.003 to 0.01 (median 0.006) and was independent of the plasma concentration within the measured concentrations of 1.7 - 9.5 µg/ml. The unbound fraction of indomethacin in plasma ranged from 0.0003 to 0.004 (median 0.0005). The CSF to protein-free plasma ratio of ketoprofen approached unity, but equilibrium between CSF and protein-free plasma samples was not reached within the 67 minute study period. Indomethacin concentrations were higher in the CSF than in protein-free plasma; only three children had a higher indomethacin concentration in protein-free plasma than in the CSF (Figure 5.3).

There was a correlation between the CSF indomethacin concentration and the child's age, younger children having higher CSF concentrations than older ones (p=0.038) (Figure 5.4). No such correlation was found between the CSF concentration of ketoprofen and the age of the children.
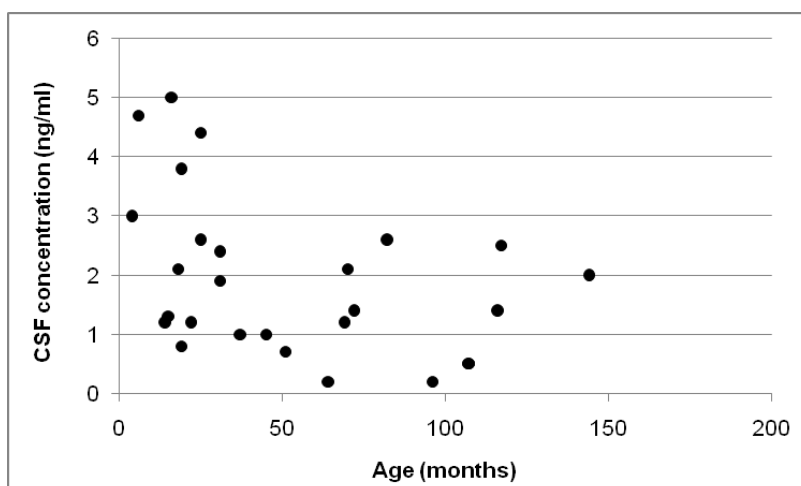
Figure 5.4. CSF concentrations of indomethacin in children aged 4-144 months.

5.3.3 Observed adverse effects

No serious adverse events were reported in the present study. In the ketoprofen study, five children reported mild pain at the injection sites. In the indomethacin study, the observed adverse effects were analysed in more detail; eleven children developed 12 non-serious adverse effects. Five children were agitated, three children vomited, one child experienced nausea, two children had abdominal pain and one child developed shivering. The observed agitation was not associated with the indomethacin CSF concentration or age. Agitation was associated with administration time (p=0.032) and occurred among children who had received indomethacin within 40 minutes prior to CSF sampling.

5.4 Discussion and conclusions

The determination of the CSF permeation of ketoprofen and indomethacin is important, because it has been proposed that the antinociceptive effects of NSAIDs could partly be due to central effects of these drugs (Reinold *et al.* 2005). In order to act centrally, a sufficient amount of the drug must enter the CNS. In the present study, the CSF permeation of ketoprofen and indomethacin was studied in children after intravenous administration of these drugs. The analytical methods used were sensitive enough to allow the determination of ketoprofen and indomethacin in the CSF, and it was shown that both ketoprofen and indomethacin are able to enter the CSF after intravenous administration. However, the CSF permeation of these two NSAIDs was limited; less than 1% of the drug plasma concentration was found in the CSF. The median lumbar CSF concentrations were 6.6 ng/ml for ketoprofen and 1.4 ng /ml for indomethacin. Whether these concentrations present in the lumbar CSF are clinically relevant will have to be evaluated in further studies. In behavioural studies in rats,

1.9 nmol (= 680 ng) of indomethacin injected intrathecally was required to produce 50% inhibition of the control response (Malmberg and Yaksh 1992).

In the studies where ketoprofen and indomethacin concentrations were measured in adults after intramuscular administration, the CSF to plasma concentration ratios were fairly similar to those obtained in the present study (Netter *et al.* 1985, Bannwarth *et al.* 1990). This suggests that the CSF permeation of ketoprofen and indomethacin in children is comparable to that of adults. In the present study, younger children appeared to have higher CSF indomethacin concentrations than older children. Although the validity of this finding must be confirmed by further experiments, it raises the possibility that the CNS permeability of indomethacin in children may be affected by age. On the contrary, no indication of any age-related CSF permeation was found for ketoprofen. However, it cannot be excluded that the age range (13-94 months) was too small to reveal any age-related effects. In general, few studies have described the effect of age on CNS permeability of different compounds. According to one study where age-related changes in the plasma to CSF equilibration half-life of acetaminophen were investigated, the BBB permeability did not seem to be increased in early infancy (van der Marel *et al.* 2003). However, more research information is needed in the area of CNS transport of drugs in children. In addition to age-dependency in BBB permeability, age-dependency in plasma protein binding might also explain the increased CSF distribution of indomethacin in younger children compared to older children. The serum concentration of albumin is lower in infants of at least up to 6 months of age than in adults (Kanakoudi *et al.* 1995), and therefore higher unbound fractions of compounds that bind to albumin can be observed in young children compared to older children or adults (Herngren *et al.* 1991). Higher unbound concentrations of compounds whose CNS distribution is restricted by plasma protein binding could increase their CNS delivery. In the present study, it could not be demonstrated that the unbound fraction of indomethacin was dependent on the child's age suggesting that age-dependency in plasma protein binding does not explain the observed age-dependency in the CSF concentrations of indomethacin.

In the present study, the CSF concentrations of ketoprofen and indomethacin were determined from the lumbar space. The drug concentrations in the lumbar CSF space are therapeutically relevant, because NSAIDs can reduce hyperalgesia by inhibiting the production of PGE_2 in the spinal cord. Furthermore, the nerves conducting noxious information from the lower part of body enter into the spinal cord at the same level. However, it has to be remembered that the lumbar concentration of the drug is not indicative of CNS permeation and disposition of the drug as a whole. Firstly, there are remarkable differences in drug concentrations between ventricular and spinal CSF, probably because of the exchange of drugs between the CSF and the cord tissue bordering the spinal subarachnoid space (Shen *et al.* 2004). Moreover, the CSF and brain parenchyma are considered to be two separate compartments e.g. due to differences between the BBB and BCSFB. Therefore, the drug concentrations determined in the present study represent only the lumbar distribution of

ketoprofen and indomethacin, and not the overall distribution to the CSF, not to mention the brain tissue distribution of these drugs.

The use of indomethacin is often associated with CNS adverse effects, such as dizziness, vertigo, headache, agitation, and even psychosis and depression (Tharumaratnam *et al*. 2000, Hoppmann *et al*. 1991, Clunie *et al*. 2003). In the present study, five out of the 31 children were agitated in the recovery room. Agitation is common in children after inhalation anaesthesia (Johr 2002), but it is rarely reported after spinal anaesthesia (Kokki and Hendolin 1995). Therefore, the observed agitation could have been an indomethacin-related CNS adverse effect (Clunie *et al*. 2003). However, in the present study the reported adverse effects were not sub-classified as being either drug-related or not. The children who experienced agitation did not have higher CSF indomethacin concentrations than those without CNS adverse effects (Table 5.2). According to our present understanding, the connection between CSF concentrations of indomethacin and indomethacin-related CNS adverse effects is rather complicated. For instance, it is not clear whether the indomethacin-related CNS adverse effects are simply due to changes in cranial blood flow or whether indomethacin has also direct effects on central neurons. If the latter is true, then the CSF permeation of indomethacin may be of clinical relevance.

In the present study, the median CSF concentration of indomethacin was reached relatively rapidly, and the sampling times chosen did not detect the initial rise in the CSF concentrations of indomethacin (Figure 5.1). On the contrary, the CSF concentrations of ketoprofen increased with time. As both NSAIDs studied in the present study achieved CSF concentrations of approximately 1% of the plasma concentrations, it seems that these two NSAIDs have relatively similar extents but different rates of CSF entry, with indomethacin being able to pass across the BCSFB more readily than ketoprofen. Lipophilicity of a drug is one of the factors which affect its ability to pass through the BBB and the BCSFB, and to enter the CNS. At the pH of 7.4, the apparent logP values of indomethacin and ketoprofen are 0.89 and -0.09, respectively (Austin *et al*. 2002). It can be hypothesized that because indomethacin is a more lipophilic molecule than ketoprofen, it enters the CSF more readily.

In studies where the antinociceptive activity of ketoprofen has been examined, it has been discovered that ketoprofen has a central analgesic effect, but rather high doses are needed to produce a reduction of nociceptive responses in electrophysiological experiments (Herrero *et al*. 1997). Gaitan *et al*. (2004) discovered that HCT-2037, a nitric oxide releasing derivative of *S*-ketoprofen, was more potent and effective as a centrally active antinociceptive agent than *S*-ketoprofen itself. It is interesting to observe that the introduction of the nitric oxide releasing group into the ketoprofen molecule makes the compound more lipophilic. The log P value of neutral ketoprofen molecule is 2.81 whereas that of HCT-2037 is 4.19 (calculated using ACD/Labs). As lipophilicity is an important factor determining the ability of a molecule to pass through the BBB and BCSFB, it is possible that the central antinociceptive actions of

HCT-2037 are improved at least partly because HCT-2037 may enter the CNS more readily than *S*-ketoprofen. However, other possibilities may explain the difference in the antinociceptive properties of HCT-2037 and in *S*-ketoprofen, e.g. different mechanism of action of HCT-2037 (Gaitan *et al*. 2004). In addition, a role for efflux mechanisms and altered interactions with efflux transporters cannot be excluded.

The BBB and the BCSFB, in addition to forming an anatomical barrier and allowing compounds to enter the CNS by passive diffusion only by the transcellular route, express various efflux transporters, with P-glycoprotein being probably the most well-known. In addition, the MRPs, OATs and OATPs have been suggested to have a role as efflux transporters at the BBB and the BCSFB (Ohtsuki *et al*. 2002, Kusuhara and Sugiyama 2004, Mori *et al*. 2004, Zhang *et al*. 2004, Kusuhara and Sugiyama 2005). As both ketoprofen (Ohtsuki *et al*. 2002) and indomethacin (Reid *et al*. 2003, Dallas *et al.* 2004, Apiwattanakul *et al*. 1999, Khamdang *et al*. 2002, Shitara *et al*. 2002) have been shown to interact with these transport proteins, a role for efflux mechanisms cannot be excluded in the CNS permeation of these drugs.

In the systemic circulation, most NSAIDs are extensively bound to plasma proteins. In the present study, the unbound fraction of ketoprofen and indomethacin in plasma was less than 1%, which is consistent with earlier reports (Netter *et al*. 1985, Bannwarth *et al*. 1990). The extensive protein binding of ketoprofen and indomethacin may restrict their permeation into the CNS, as it is believed that only unbound drug can pass through biological membranes (Rowland and Tozer 1995). Since the CSF space is rather difficult to access, the estimation of the CSF concentration of the drug based on the unbound plasma concentration would be advantageous. Netter *et al.* (1985) have reported that the CSF levels of ketoprofen were equivalent to the protein-free serum levels 2-3 hours after intramuscular administration. Unfortunately in the present study the study period of 67 minutes was too short to allow determination of reliable t_{max} and C_{max} values in CSF, and further, to evaluate the possible equilibrium between the CSF and unbound plasma levels of ketoprofen. However, the CSF levels increased with time, resulting in unbound plasma to CSF ratio which approached unity.

In the case of indomethacin, drug concentrations were higher in the CSF than in protein-free plasma; only three children had a higher indomethacin concentration in protein-free plasma than in the CSF (Table 5.2). In the present study, the CSF was regarded as practically protein-free, as the albumin concentration in the CSF is only 0.4% of that present in serum (Davson and Segal 1996). According to Muller *et al*. (1991), indomethacin binds to proteins present not only in the plasma but also in the CSF, the mean binding percentage of indomethacin being 40%. Therefore, as we only measured the total CSF concentration of indomethacin, it is possible that we observed higher indomethacin concentrations in the CSF than in protein-free plasma. Thus, it is important to determine the unbound drug concentration from the CSF, especially if the drug is extensively bound to proteins. There are some studies where total and

unbound CSF drug concentrations have been determined, for instance after propofol administration. While the unbound fraction of propofol in plasma was 1%, in the CSF the unbound fraction was 31% (Dawidowicz *et al*. 2003). Additionally, total CSF concentrations of propofol were much higher than the unbound plasma concentrations. Protein binding may have a notable influence on the drug CSF concentration, particularly when the CSF is sampled at the lumbar space, since the protein concentrations are higher in lumbar CSF than in ventricular or cisternal CSF (Shen *et al*. 2004). Given the possible contribution of protein binding within the (lumbar) CSF one has to be careful in making predictions of CSF concentrations based on unbound plasma concentrations. Other reasons that might explain higher CSF concentrations of a compound relative to plasma unbound concentrations can be active transport mechanisms at the BCSFB or differences in the pH of plasma versus CSF (Rowland and Tozer 1995).

In conclusion, the present study demonstrated that ketoprofen and indomethacin are able to permeate the CSF of children after intravenous administration. The determination of the CSF concentrations of ketoprofen and indomethacin was achieved with a sensitive GC-MS method. In terms of evaluating the extent of CSF permeation based on total plasma concentration, the CSF permeation of ketoprofen and indomethacin was rather limited after intravenous administration since less than 1% of the total plasma drug concentration was detected in the CSF. Whether this is sufficient to mediate any central antinociceptive effects in the lumbar space, or be involved in other central mechanisms of action remains to be clarified.

The extent of the CSF permeation of ketoprofen and indomethacin in children was rather similar to the CSF permeation of these drugs reported in adults. In the case of indomethacin, the present study suggests that CSF distribution may be affected by age as the younger children appeared to have higher indomethacin CSF concentrations than the older children. This finding, however, needs to be confirmed in future studies.

No equilibrium between the CSF concentrations and the unbound drug concentrations in the plasma was observed in the present study, suggesting that the unbound plasma concentration may not be indicative of the CSF concentration of ketoprofen and indomethacin. When the CSF concentration of ketoprofen was studied, the C_{max} and t_{max} values could not be assessed with the study protocol used, but the CSF to unbound plasma concentration ratio approached unity. On the contrary, the CSF total concentrations of indomethacin were higher than those in protein-free plasma. This might be due to protein binding of indomethacin in the CSF and emphasizes the importance of the determination of unbound drug concentration from the CSF for those drugs that are highly bound to proteins.

5.5 References

Apiwattanakul, N., Sekine, T., Chairoungdua, A., Kanai, Y., Nakajima, N., Sophasan, S., Endou, H. Transport properties of nonsteroidal anti-inflammatory drugs by organic anion transporter 1 expressed in Xenopus laevis oocytes. Mol Pharmacol 55: 847-54, 1999.

Austin, R. P., Barton, P., Cockroft, S. L., Wenlock, M. C., Riley, R. J. The influence of nonspecific microsomal binding on apparent intrinsic clearance, and its prediction from physicochemical properties. Drug Metab Dispos 30: 1497-503, 2002.

Bannwarth, B., Netter, P., Lapicque, F., Pere, P., Thomas, P., Gaucher, A. Plasma and cerebrospinal fluid concentrations of indomethacin in humans. Relationship to analgesic activity. Eur J Clin Pharmacol 38: 343-6, 1990.

Clunie, M., Crone, L. A., Klassen, L., Yip, R. Psychiatric side effects of indomethacin in parturients. Can J Anaesth 50: 586-8, 2003.

Daher, J. B., Tonussi, C. R. A spinal mechanism for the peripheral anti-inflammatory action of indomethacin. Brain Res 962: 207-12, 2003.

Dallas, S., Schlichter, L., Bendayan, R. Multidrug resistance protein (MRP) 4- and MRP 5-mediated efflux of 9-(2-phosphonylmethoxyethyl)adenine by microglia. J Pharmacol Exp Ther 309: 1221-9, 2004.

Dawidowicz, A. L., Kalitynski, R., Fijalkowska, A. Free and bound propofol concentrations in human cerebrospinal fluid. Br J Clin Pharmacol 56: 545-50, 2003.

Davson, H., Segal, M. B. Physiology of the CSF and the blood-brain barriers, New York, CRC Press, Inc, 1996.

Dittrich, P., Kohler, G., Primbs, P., Kukovetz, W. R. Pharmacokinetics of indomethacin i.m. in blood, synovial fluid, synovial membrane, muscle, fat, bone, and spinal fluid. Eur J Rheumatol Inflamm 7: 45-50, 1984.

Gaitan, G., Ahuir, F. J., Del Soldato, P., Herrero, J. F. Comparison of the antinociceptive activity of two new NO-releasing derivatives of the NSAID S-ketoprofen in rats. Br J Pharmacol 143: 533-40, 2004.

Herngren, L., Lundberg, B., Nergård, A. Pharmacokinetics of total and free valproic acid during monotherapy in infants. J Neurol 238: 315-9, 1991.

Herrero, J. F., Parrado, A., Cervero, F. Central and peripheral actions of the NSAID ketoprofen on spinal cord nociceptive reflexes. Neuropharmacology 36: 1425-31, 1997.

Hoppmann, R. A., Peden, J. G., Ober, S. K. Central nervous system side effects of nonsteroidal anti-inflammatory drugs. Aseptic meningitis, psychosis, and cognitive dysfunction. Arch Intern Med 151: 1309-13, 1991.

Johr, M. Postanaesthesia excitation. Paediatr Anaesth 12: 293-5, 2002.

Kanakoudi, F., Drossou, V., Tzimouli, V., Diamanti, E., Konstantinidis, T., Germenis, A., Kremenopoulos, G. Serum concentrations of 10 acute-phase proteins in healthy term and preterm infants from birth to age 6 months. Clin Chem 41: 605-8, 1995.

Khamdang, S., Takeda, M., Noshiro, R., Narikawa, S., Enomoto, A., Anzai, N., Piyachaturawat, P., Endou, H. Interactions of human organic anion transporters and human organic cation transporters with nonsteroidal anti-inflammatory drugs. J Pharmacol Exp Ther 303: 534-9, 2002.

Kokki, H., Hendolin, H. Comparison of spinal anaesthesia with epidural anaesthesia in paediatric surgery. Acta Anaesthesiol Scand 39: 896-900, 1995.

Kokki, H., Karvinen, M., Jekunen, A. Diffusion of ketoprofen into the cerebrospinal fluid of young children. Paediatr Anaesth, 12**,** 313-6, 2002.

Kusuhara, H., Sugiyama, Y. Efflux transport systems for organic anions and cations at the blood-CSF barrier. Adv Drug Deliv Rev 56: 1741-63, 2004.

Kusuhara, H., Sugiyama, Y. Active efflux across the blood-brain barrier: role of the solute carrier family. NeuroRx 2: 73-85, 2005.

Leis, H. J., Leis, M., Windischhofer, W. Stable isotope labelling and determination of ketroprofen in human plasma and urine by gas chromatography/negative ion chemical ionization mass spectrometry. J Mass Spectrom 31: 486-92, 1996.

Malmberg, A. B., Yaksh, T. L. Antinociceptive actions of spinal nonsteroidal anti-inflammatory agents on the formalin test in the rat. J Pharmacol Exp Ther 263: 136-46, 1992.

Maunuksela, E. L., Olkkola, K. T., Korpela, R. Does prophylactic intravenous infusion of indomethacin improve the management of postoperative pain in children? Can J Anaesth 35: 123-7, 1988.

Mori, S., Ohtsuki, S., Takanaga, H., Kikkawa, T., Kang, Y. S., Terasaki, T. Organic anion transporter 3 is involved in the brain-to-blood efflux transport of thiopurine nucleobase analogs. J Neurochem 90: 931-41, 2004.

Muller, N., Lapicque, F., Monot, C., Payan, E., Gillet, P., Bannwarth, B., Netter, P. Protein binding of indomethacin in human cerebrospinal fluid. Biochem Pharmacol 42: 799-804, 1991.

Netter, P., Lapicque, F., Bannwarth, B., Tamisier, J. N., Thomas, P., Royer, R. J. Diffusion of intramuscular ketoprofen into the cerebrospinal fluid. Eur J Clin Pharmacol 29: 319-21, 1985.

Ohtsuki, S., Asaba, H., Takanaga, H., Deguchi, T., Hosoya, K., Otagiri, M., Terasaki, T. Role of blood-brain barrier organic anion transporter 3 (OAT3) in the efflux of indoxyl sulfate, a uremic toxin: its involvement in neurotransmitter metabolite clearance from the brain. J Neurochem 83: 57-66, 2002.

Ossipov, M. H., Jerussi, T. P., Ren, K., Sun, H., Porreca, F. Differential effects of spinal (R)-ketoprofen and (S)-ketoprofen against signs of neuropathic pain and tonic nociception: evidence for a novel mechanism of action of (R)-ketoprofen against tactile allodynia. Pain 87: 193-9, 2000.

Reid, G., Wielinga, P., Zelcer, N., van der Heijden, I., Kuil, A., de Haas, M., Wijnholds, J., Borst, P. The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs. Proc Natl Acad Sci U S A 100: 9244-9, 2003.

Reinold, H., Ahmadi, S., Depner, U. B., Layh, B., Heindl, C., Hamza, M., Pahl, A., Brune, K., Narumiya, S., Muller, U., Zeilhofer, H. U. Spinal inflammatory hyperalgesia is mediated by prostaglandin E receptors of the EP2 subtype. J Clin Invest 115: 673-9, 2005.

Rowland, M., Tozer, T. N. Clinical Pharmacokinetics, Concepts and Applications, Philadelphia, Lippincott Williams, Wilkins, 1995.

Shen, D. D., Artru, A. A., Adkison, K. K. (2004) Principles and applicability of CSF sampling for the assessment of CNS drug delivery and pharmacodynamics. Adv Drug Deliv Rev, 56**,** 1825-57.

Shitara, Y., Sugiyama, D., Kusuhara, H., Kato, Y., Abe, T., Meier, P. J., Itoh, T., Sugiyama, Y. Comparative inhibitory effects of different compounds on rat oatpl (slc21a1)- and Oatp2 (Slc21a5)-mediated transport. Pharm Res 19**:** 147-53, 2002.

Tharumaratnam, D., Bashford, S., Khan, S. A. Indomethacin induced psychosis. Postgrad Med J 76: 736-7, 2000.

van der Marel, C. D., Anderson, B. J., Pluim, M. A., de Jong, T. H., Gonzalez, A., Tibboel, D. Acetaminophen in cerebrospinal fluid in children. Eur J Clin Pharmacol 59: 297-302, 2003.

Van Overmeire, B., Chemtob, S. The pharmacologic closure of the patent ductus arteriosus. Semin Fetal Neonatal Med 10**:** 177-84, 2005.

Zhang, Y., Schuetz, J. D., Elmquist, W. F., Miller, D. W. Plasma membrane localization of multidrug resistance-associated protein homologs in brain capillary endothelial cells. J Pharmacol Exp Ther 311: 449-55, 2004.

6 GENERAL DISCUSSION

In modern CNS drug development it is becoming clear that one has to take into account several properties in order to have a successful compound. A good CNS drug needs to possess not only high permeability across the BBB but also low potential to be a substrate for active efflux mechanisms and appropriate partitioning of the unbound drug between blood and brain (Jeffrey and Summerfield 2007). Furthermore, one has to remember that the rate and extent of CNS distribution are separate processes and, for instance, a slow rate of transport across the blood-CNS barriers does not necessarily lead to a low level of drug in the CNS, and vice versa (Hammarlund-Udenaes 2000).

The extent of CNS permeation is often quantified as the total brain to total plasma concentration ratio. However, there is a growing awareness that the total brain to total plasma concentration ratio, which is dependent upon several factors including protein binding in plasma and brain, BBB uptake, influx and efflux transport mechanisms, metabolism and bulk flow, is of limited value in the determination of CNS permeation. It has been suggested that the use of unbound drug concentrations both in plasma and in brain tissue or the use of BBB PA values could provide more accurate information about CNS permeation of compounds (Jeffrey and Summerfield 2007, Liu *et al*. 2005, Pardridge 2004).

The aim of the present study was to obtain a better understanding of the CNS permeation and distribution properties of three traditional NSAIDs ibuprofen, indomethacin and ketoprofen. It was hypothesized that their CNS permeation would be limited after systemic administration. The extent of the CNS permeation was evaluated based on the ratio between the total brain or the total CSF concentration and the plasma concentration. Indeed based on the low brain or CSF to plasma ratios determined in this study, it can be concluded that the CNS permeation of ibuprofen, indomethacin and ketoprofen is limited. Furthermore, it is suggested that the CNS permeation of indomethacin is limited by its extremely high plasma protein binding although there might be also an efflux system involved. However, as discussed above, the use of the total brain to total plasma concentration ratio has its own limitations in the evaluation of CNS permeation. On the contrary, the CSF is often regarded as almost protein-free which makes the CSF to plasma ratio – and the evaluation of CSF permeation more accurate. However, the CSF concentration of a compound is not necessarily equal to the concentration in the brain parenchyma as they are regarded as two distinct compartments (de Lange and Danhof 2002).

The constant *in vivo* infusion method and the *in situ* brain perfusion method in rats proved to be very useful in the determination of CNS permeation. With slight modifications, a broad range of information can be obtained using these techniques, including the evaluation of the effect of efflux inhibitors and plasma protein binding on the brain permeation of compounds. The incorporation of the determination of unbound drug concentrations both in plasma and in

brain tissue would make these models extremely accurate. The determination of ibuprofen, indomethacin and ketoprofen from brain tissue homogenate, CSF, plasma and protein-free plasma was conducted by using several sample pre-treatment and analytical methods. In particular, the low drug concentrations in the human CSF and in the protein-free plasma demand extreme sensitivity from the assay methods. The significance of the sensitive analytical methods is likely to increase if the CNS permeation of compounds is to be determined by assaying the unbound concentrations in plasma, CSF and brain tissue.

6.1 References

de Lange, E. C., Danhof, M. Considerations in the use of cerebrospinal fluid pharmacokinetics to predict brain target concentrations in the clinical setting: implications of the barriers between blood and brain. Clin Pharmacokinet 41: 691-703, 2002.

Hammarlund-Udenaes, M. The use of microdialysis in CNS drug delivery studies. Pharmacokinetic perspectives and results with analgesics and antiepileptics. Adv Drug Deliv Rev 45: 283-94, 2000.

Jeffrey, P., Summerfield, S. G. Challenges for blood-brain barrier (BBB) screening. Xenobiotica 37: 1135-51, 2007.

Liu, X., Smith, B. J., Chen, C., Callegari, E., Becker, S. L., Chen, X., Cianfrogna, J., Doran, A. C., Doran, S. D., Gibbs, J. P., Hosea, N., Liu, J., Nelson, F. R., Szewc, M. A., Van Deusen, J. Use of a physiologically based pharmacokinetic model to study the time to reach brain equilibrium: an experimental analysis of the role of blood-brain barrier permeability, plasma protein binding, and brain tissue binding. J Pharmacol Exp Ther 313: 1254-62, 2005.

Pardridge, W. M. Log(BB), PS products and in silico models of drug brain penetration. Drug Discov Today 9: 392-3, 2004.

7 CONCLUSIONS

In the present study, the CNS permeation of three traditional NSAIDs ibuprofen, indomethacin and ketoprofen was evaluated. The following conclusions can be made:

- Ibuprofen and indomethacin permeated poorly into the rat CNS after intravenous administration, as indicated by the low total brain to total plasma concentration ratio of 0.02 for ibuprofen and 0.01 for indomethacin. The unbound fraction of indomethacin permeated quite readily into the CNS during *in situ* brain perfusion and based on the total brain to unbound plasma concentration ratio after intravenous infusion.

- The brain permeation of indomethacin could be increased by co-administration of probenecid. This suggests that brain permeation of indomethacin may be influenced by a probenecid-sensitive efflux mechanism.

- An increase in the unbound fraction enhanced the brain permeation of indomethacin.

- The CSF permeation of ketoprofen and indomethacin was limited after intravenous administration in children since only less than 1% of the plasma drug concentration was detected in the CSF. CSF to unbound plasma concentration ratios of ketoprofen were less than unity within the sampling period, whereas the majority of the CSF to unbound plasma concentration ratios of indomethacin were above unity.

- Ketoprofen and indomethacin seem to have different rates of CSF entry, with indomethacin being able to pass across the BCSFB more readily than ketoprofen when evaluated by the time when the maximum CSF concentrations were observed.

Kuopio University Publications A. Pharmaceutical Sciences

A 100. Toropainen, Elisa. Corneal epithelial cell culture model for pharmaceutical studies.
2007. 81 p. Acad. Diss.

A 101. Mannila, Janne. Cyclodextrins in intraoral delivery of delta-9-tetrahydrocannabinol and cannabidiol.
2007. 90 p. Acad. Diss.

A 102. Männistö, Marjo. Polymeric carriers in non-viral gene delivery: a study of physicochemical properties and biological activity in human RPE cell line.
2007. 65 p. Acad. Diss.

A 103. Mauriala, Timo. Development of LC-MS methods for quantitative and qualitative analyses of endogenous compounds, drugs, and their metabolities to support drug discovery programs.
2007. 126 p. Acad. Diss.

A 104. Kumpulainen, Hanna. Novel prodrug structures for improved drug delivery.
2007. 129 p. Acad. Diss.

A 105. Korjamo, Timo. Improvement of the Caco-2 permeability model by genetic and hydrodynamic modifications.
2008. 134 p. Acad. Diss.

A 106. Pappinen, Sari. The organotypic culture of rat epidermal keratinocytes (ROC) in pharmaceutical and chemical testing.
2008. 83 p. Acad. Diss.

A 107. Mönkkönen, Kati. $G\alpha i_2$ in ciliated tissues: physiological role in regulation of ependymal ciliary function and characteristics in human female reproductive tissues.
2008. 85 p. Acad. Diss.

A 108. Matilainen, Laura. Cyclodextrins in peptide deliverty: in vitro studies for injectable and inhaled formulations.
2008. 144 p. Acad. Diss.

A 109. Lääkepäivät : lääkkeitä myös terveille? 25.-26.4.2008, Kuopio.
2008. 120 p. Abstracts.

A 110. Myöhänen, Timo. Distribution of prolyl oligopeptidase and its colocalizations with neurotransmitters and substrates in mammalian tissues.
2008. 101 p. Acad. Diss.

A 111. Toropainen, Tarja. Cyclodextrins and their solid-state complexes: studies for pulmonary drug delivery.
2008. 136 p. Acad. Diss.

A 112. Määttä, Juha. Cytokine, chemokine, and chemokine receptor expression in RAW 264.7 mouse macrophages and in the lungs of mice after exposure to wood dust.
2008. 69 p. Acad. Diss.

A 113. Kääriäinen, Tiina. Interaction of the dopaminergic and serotonergic systems in rat brain: studies in parkinsonian models and brain microdialysis.
2008. 128 p. Acad. Diss.

A 114. Kiviranta, Päivi H. Design and synthesis of silent information regulator human type 2 (SIRT2) inhibitors. 2008. 148 p. Acad. Diss.