Blood-Brain Barrier Transport

Investigation of Active Efflux using Positron Emission Tomography and Modelling Studies

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Abstract

This thesis examines the transport of exogenous molecules across the blood-brain barrier (BBB), focusing on active efflux, using positron emission tomography (PET), computer simulation and modelling. P-glycoprotein (P-gp) inhibition was studied using [11C]verapamil and [11C]hydroxyurea was investigated as a new marker for active efflux transport. Simulations were carried out to explore the importance of the efflux transporter location in the BBB. Brain concentrations of [11C]verapamil, [11C]GR205171 and [18F]altanserin were compared in various laboratory animal species and in humans.

A central aspect of the studies has been the novel combination of dynamic PET imaging of the brain pharmacokinetics of a labelled drug, administered through an exponential infusion scheme allowing time-resolved consequence analysis of P-gp inhibition, and mathematical modelling of the obtained data. The methods are applicable to drugs under development and can be used not only in rodents but also in higher species, potentially even in humans, to investigate the effects of P-gp or other transporters on drug uptake in the brain.

The inhibition of P-gp by cyclosporin A (CsA) and the subsequent change in brain concentrations of [11C]verapamil occurred rapidly in the sense that [11C]verapamil uptake increased rapidly after CsA administration but also in the sense that the increased uptake was rapidly reversible. The P-gp inhibition was best described by an inhibitory indirect effect model in which CsA decreased the transport of [11C]verapamil out of the brain. The model indicated that approximately 90% of the transport of [11C]verapamil was P-gp-mediated. The low brain concentrations of [11C]hydroxyurea appeared to be a result of slow transport across the BBB rather than active efflux. This exemplifies why the extent and the rate of brain uptake should be approached as two separate phenomena. The brain-to-plasma concentration ratios for the three studied radiotracers differed about 10-fold between species, with lower concentrations in rodents than in humans, monkeys and pigs. The increase in brain concentrations after P-gp inhibition was somewhat greater in rats than in the other species.

The findings demonstrate a need to include the dynamics of efflux inhibition in the experimental design and stress the importance of the choice of species in preclinical studies of new drug candidates.

Keywords: pharmacokinetics, P-glycoprotein, blood-brain barrier, modelling, PET, active efflux, species differences, [11C]verapamil, drug development

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List of papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


V  Syvänen S., Lindhe Ö., Palner M., Kornum B.R., Rahman O., Långström B., Knudsen G.M. and Hammarlund-Udenaes M. Species differences in blood-brain barrier transport of three PET radiotracers with emphasis on P-glycoprotein and plasma protein binding. (manuscript)

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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADME</td>
<td>adsorption, distribution, metabolism and elimination of a drug</td>
</tr>
<tr>
<td>AIC</td>
<td>akaike information criterion</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>$A_{\text{tot,br}}$</td>
<td>total (unbound, bound, intracellular etc) amount of drug in the brain (mg/g-brain)</td>
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<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>Bcrp</td>
<td>breast cancer resistance protein</td>
</tr>
<tr>
<td>CL</td>
<td>systemic clearance (mL/min)</td>
</tr>
<tr>
<td>CL_pass</td>
<td>unbound passive clearance (μL/min/g-brain)</td>
</tr>
<tr>
<td>CL_1</td>
<td>clearance by influx hindrance (μL/min/g-brain)</td>
</tr>
<tr>
<td>CL_2</td>
<td>clearance by efflux enhancement at the luminal membrane (μL/min/g-brain)</td>
</tr>
<tr>
<td>CL_3</td>
<td>clearance by efflux enhancement at the abluminal membrane (μL/min/g-brain)</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CsA</td>
<td>cyclosporin A</td>
</tr>
<tr>
<td>CsA_bl</td>
<td>cyclosporin A concentration in blood (μg/mL)</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>CT</td>
<td>computerized tomography</td>
</tr>
<tr>
<td>C_bl</td>
<td>drug concentration in blood</td>
</tr>
<tr>
<td>C_ub,bl</td>
<td>concentration of unbound drug in blood</td>
</tr>
<tr>
<td>C_ub,br</td>
<td>concentration of unbound drug in brain</td>
</tr>
<tr>
<td>C_ub,cc</td>
<td>concentration of unbound drug in endothelial cells</td>
</tr>
<tr>
<td>C_ub,ss,bl</td>
<td>concentration of unbound drug in blood at steady-state</td>
</tr>
<tr>
<td>C_ub,ss,br</td>
<td>concentration of unbound drug in brain at steady-state</td>
</tr>
<tr>
<td>ECF</td>
<td>extracellular fluid</td>
</tr>
<tr>
<td>$E_{\text{kin}}$</td>
<td>the effect of CsA on $k_{\text{in}}$</td>
</tr>
<tr>
<td>$E_{\text{kout}}$</td>
<td>the effect of CsA on $k_{\text{out}}$</td>
</tr>
<tr>
<td>$E_{\text{max}}$</td>
<td>maximum change in $k_{\text{in}}$ or $k_{\text{out}}$</td>
</tr>
<tr>
<td>$\eta_i$</td>
<td>inter-animal variability</td>
</tr>
<tr>
<td>$f_p$</td>
<td>fraction of drug not bound to plasma protein</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>sigmoidicity factor in effect models</td>
</tr>
<tr>
<td>g-brain</td>
<td>one gram of brain tissue</td>
</tr>
<tr>
<td>IC_50</td>
<td>concentration required to achieve half the maximum change in $k_{\text{in}}$ or $k_{\text{out}}$</td>
</tr>
<tr>
<td>ISF</td>
<td>interstitial fluid</td>
</tr>
<tr>
<td>$J_{\text{max}}$</td>
<td>maximal active transport capacity elimination constant (ng/min/g-brain)</td>
</tr>
<tr>
<td>k</td>
<td>rate constant (/min)</td>
</tr>
<tr>
<td>$k_{\text{el}}$</td>
<td>elimination constant (/min)</td>
</tr>
</tbody>
</table>
The page contains a list of abbreviations and their definitions. Here is a structured representation of the information:

- $k_{in}$: rate constant for transport of drug into the brain (/min)
- $k_{out}$: rate constant for transport of drug out from the brain (/min)
- $K_P$: ratio of total brain to total plasma concentration
- $K_{p,u}$: ratio of total concentration in brain to concentration of unbound drug in plasma
- $K_{p,uu}$: ratio of concentration of unbound drug in brain to that of unbound drug in plasma
- $K_{i}$: concentration of unbound drug at 50% of $J_{max}$
- MDR1: multidrug resistance gene coding for P-gp
- Mrp: multidrug resistance protein
- OFV: objective function value
- Oat: organic anion transporter
- Oatp: organic anion transporting polypeptide
- PET: positron emission tomography
- P-gp: P-glycoprotein
- $P_{i}$: pharmacokinetic parameter in an individual animal
- $P_{pop}$: pharmacokinetic parameter in a typical animal
- PS: permeability surface area product ($\mu$L/min/g-brain)
- SUV: standardized uptake value
- $R_{0}$: infusion rate (mg/min)
- $R_{buffer}$: radioactivity in buffer in equilibrium dialysis
- $R_{plasma}$: radioactivity in plasma in equilibrium dialysis
- ROI: region of interest (in PET images)
- $V$: distribution volume (mL)
- $V_{bl}$: volume of blood in the brain tissue (mL/g-brain)
- $V_{d}$: “tissue distribution volume”; PET nomenclature for tissue-to-plasma partitioning coefficient; equal to $K_p$
- VER$_{pl}$: [$^{11}$C]verapamil concentrations in the plasma (Bq/mL)
- VER$_{br}$: [$^{11}$C]verapamil concentrations in the brain (Bq/mL)
- $V_{u,bl}$: distribution volume of unbound drug in blood (mL/g-brain)
- $V_{u,br}$: distribution volume of unbound drug in brain (mL/g-brain)
- $V_{u,cc}$: distribution volume of unbound drug in endothelial cells (mL/g-brain)
Introduction

Many commonly used drugs are intended for treatment of brain disorders or diseases such as depression, schizophrenia, anxiety, epilepsy, Parkinson’s and Alzheimer’s diseases and brain tumours. These drugs elicit their effect by interaction with targets inside the brain. The targets can be receptors, transporters, enzymes or other proteins. Most drugs are administered orally and then absorbed from the gut to the blood while some drugs are administered directly into the blood stream as an intravenous injection. Regardless of whether the drugs are orally or systemically administered, they must pass the blood-brain barrier (BBB), which separates the brain from the blood, before they can act on their final targets. The BBB consists of a tightly connected cell layer with associated properties which can sometimes hinder the passage of drug molecules from the blood to the brain. For example, the BBB contains transporter proteins that actively transport some drug molecules from the brain back to the blood.

The effect of a drug aimed for a target inside the brain is usually related to the concentration of the drug inside the brain; this concentration is understandably difficult to measure. It is often not possible to take a tissue sample from a living brain, especially in human subjects. Preclinical studies in animals can be used to estimate the concentration-time profiles of drugs in the brain, but the results cannot always predict the pharmacokinetics in humans.

Hence, the development of new drugs that elicit their effect inside the brain is complicated because of both the protective nature of the BBB and the technical difficulties in studying drug concentrations at the effect site in humans. With positron emission tomography (PET), it is possible to measure drug concentrations non-invasively in organs such as the brain. The method has therefore gained an increasingly important role in drug development processes, especially in the development of drugs aimed for targets in the brain [1-3].

This thesis deals with the transport of drug molecules across the BBB and the effects of active efflux. New methods of studying BBB transport using PET are presented, along with mathematical models to describe this transport.
The blood-brain barrier

The BBB and its transport properties can have a profound influence on the uptake of drugs into the brain, and subsequently on their central effects. In contrast to many other capillary membranes in the body, such as the epithelial cell layer lining the lumen of the digestive tract, the endothelial cells of the BBB are connected to each other by so-called tight junctions, as shown in Figure 1. The tight junctions between the endothelial cells of the BBB force drugs to penetrate through rather than between the cells. Lipophilic drugs often penetrate the biological membranes in the body faster than hydrophilic drugs because of the lipophilic nature of the endothelial cell membrane. The effect of the tight junctions in the BBB therefore accentuates the differences in penetration between lipophilic and hydrophilic drugs compared with other capillary membranes in the body. The endothelial cells also contain drug-metabolizing enzymes and these may further restrict the entry of drug molecules into the brain.

![Figure 1](image)

**Figure 1.** The two membranes of the blood-brain barrier are called the luminal and abluminal membranes. The tight junctions that connect the endothelial cells of the blood-brain barrier to each other restrict the passage of drug molecules between the cells. Instead, drug molecules have to cross both membranes of the blood-brain barrier by passive diffusion or active transport. Passive diffusion is the random movement of molecules in the direction of the lower concentration. Active transporters can move molecules against the concentration gradient by different types of mechanisms.

The BBB comprises luminal and abluminal membranes, facing capillary blood and brain interstitial fluid, respectively, Figure 1. Both of these membranes contain active efflux transporters which lower the concentrations of drugs or endogenous substances in the brain. The efflux transporters, which have an important role in protecting the brain from potentially harmful substances such as toxins, have very wide substrate specificity and are thus able
to protect the brain from toxins with different molecular structures. Unfortunately, they can therefore also hinder drug molecules from entering the brain, thus preventing the potentially beneficial effects. On the other hand, the transporters can help to reduce the central side effects of drugs that are used for peripheral treatment by hindering them from entering the brain. There are two possible mechanisms behind the lowering of drug concentrations in the brain by transporters, influx hindrance decreases the influx of drug molecules into the brain and efflux enhancement increases the efflux out of the brain.

Transporters

The best known and, according to current understanding, the most important efflux transporter of exogenous substances is P-glycoprotein (P-gp), an ATP-dependent 170-180 kDa transmembrane glycoprotein. P-gp is present at the luminal membrane of the BBB [4,5]. It consists of 1280 amino acids with 12 transmembrane domains divided into two parts. Each half contains a substrate-binding domain and an ATP-binding domain. A typical P-gp substrate is a lipophilic, amphipathic molecule with a planar ring system, a positive charge at pH 7.4 and a molecular weight above 400 [6]. However, this is by no means a rule, as it has been shown that P-gp also transports neutral, negatively charged, hydrophilic and small molecules. Seelig et al. have proposed that the only requirement for a P-gp substrate is a degree of hydrogen bonding in a distinct spatial arrangement and a planar aromatic region [7]. According to this theory, the binding to and dissociation from P-gp would depend solely on the strength of the hydrogen bonds formed between P-gp and its substrates [8].

The action of P-gp has been described by the so-called “vacuum cleaner model” [9-11]. According to this model P-gp pumps substrates directly from the luminal membrane back to the blood. The vacuum-cleaner mode of action will result in transport of the drug back from the endothelial cells to the blood before it reaches the brain. P-gp is thought to extrude its substrates via both influx hindrance and efflux enhancement, perhaps depending on the substrate and its binding site [10,12,6]. Experiments have indicated that P-gp might have more than one binding site. Binding of vinblastine, a P-gp substrate, to P-gp is inhibited by both vincristine and daunorubicin, indicating the same binding site [13]. However, two other substrates, colchicine and actinomycin D, do not compete for vinblastine binding sites, which suggests the existence of two substrate binding sites [13,14]. Further, some inhibitors are solely inhibitors and not substrates for P-gp, a finding that may suggest a third binding site [15].

Three variants of the P-gp encoding gene have been characterized in rodents (mdr1a, mdr1b and mdr3) and two in humans (MDR1 and MDR2).
The human MDR1 is found in the BBB, lung, intestine, adrenal gland, kidney, liver, testis and placenta [16,17]. The rodent mdr1a is expressed at high levels in the intestine, liver and testis and is also found in the lung and BBB, while the rodent mdr1b is found in the adrenal gland and ovary. Both the rodent mdr1 and the human MDR1 are associated with multidrug resistance. In contrast, MDR2 and mdr2 do not seem to contribute to multidrug resistance; they are located in the hepatocytes in the liver and secrete phospholipids across the hepatocyte membranes into bile [16,18]. P-gp shows a large degree of homology across species with respect to amino acid content. The amino acid sequences of P-gp are compared for mouse, rat, guinea pig, dog, rhesus monkey, chimpanzee and human in Table 1.

Verapamil, a calcium-channel blocker, is a well-known substrate for P-gp. Verapamil labelled with $^{11}$C, $^{[11]}$Cverapamil, is the most commonly used radiotracer for PET studies of the P-gp function [19-27]. Verapamil has also been used in *in vitro* studies as a P-gp inhibitor. The *in vitro* IC$_{50}$ of verapamil for P-gp inhibition is in the range of 1-5 μM [28,29]. However, verapamil is lethal at doses lower than those needed to inhibit P-gp and can therefore not be used as an inhibitor of P-gp in *in vivo* studies. Cyclosporin A (CsA) is a potent immunosuppressant and is used clinically before and after organ transplantations. CsA is also a very potent *in vitro* and *in vivo* inhibitor of P-gp as well as being a substrate [30-32]. The *in vitro* IC$_{50}$ of CsA for P-gp inhibition is in the range of 0.13-3.5 μM (0.16-4.2 μg/mL) [33-35,29].

As mentioned above, there are other efflux transporters. These include the organic anion transporter 3 (Oat3), located on the abluminal side of the BBB; multidrug resistance proteins (Mrp) and organic anion transporting polypeptide 2 (Oatp2), located at both the luminal and abluminal membranes; and the breast cancer resistance protein (Bcrp), located at the luminal membrane [36-42]. It has been speculated that abluminal transporters like Oat3 require assistance from a luminal transporter to extrude substances from the brain all the way to the blood [43,44]. Transporters that actively pick up molecules and transport them into the brain, i.e. active influx transporters, are also present at the BBB. Substrates for such transporters include glucose and amino acids. In addition, some investigations have indicated that drugs can be actively transported into the brain [45-47].

Active transporters play a quantitative role in the entry of some drugs to the brain. It is thought that these transporters could be saturated by high concentrations of the substrate, and modulated by drugs or the physiological conditions.
Table 1. Comparison of amino acid sequences of P-glycoprotein in human, chimpanzee, rhesus monkey, dog, guinea pig, rat and mouse

<table>
<thead>
<tr>
<th>P-glycoprotein(^b)</th>
<th>Percent of amino acid homology(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Chimpanzee Rhesus monkey Dog Guinea pig(^c) Rat Mouse</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>87 80 85 83 82 93 100</td>
</tr>
<tr>
<td>Rat</td>
<td>85 83 88 83 80 100</td>
</tr>
<tr>
<td>Guinea Pig(^c)</td>
<td>82 82 82 82 100</td>
</tr>
<tr>
<td>Dog</td>
<td>87 89 87 100</td>
</tr>
<tr>
<td>Rhesus monkey</td>
<td>93 94 100</td>
</tr>
<tr>
<td>Chimpanzee</td>
<td>97 100</td>
</tr>
<tr>
<td>Human</td>
<td>100</td>
</tr>
</tbody>
</table>

\(^a\) The percentage identity calculated using Ensembl 45 protein sequences [48] and Blast 2 sequence comparison [49].
\(^b\) P-glycoprotein, transcribed from MDR1 (human, chimpanzee, rhesus monkey, dog, guinea pig) and mdr1a (rat and mouse) genes.
\(^c\) The whole gene sequence is not yet available. The amino acid sequences were predicted by projecting human Ensembl transcripts through a BLASTZ DNA alignment to the human genome.

Quantification of brain uptake

There is some confusion regarding what can be considered to be optimal drug brain uptake. Drugs administered on an on-going basis, i.e. most clinically used drugs, do not necessarily have to be transported into the brain at a fast rate. For these compounds, it is the steady-state or maximum and trough concentrations, i.e. the extent of brain uptake, that are of interest. However, for a PET radiotracer, a fast brain uptake is important, as the radiotracer need to enter the brain during the scanning time, which is limited by the decay half-life of the chosen positron-emitting isotope. Hence, the uptake of drugs into the brain is associated with two distinct issues: the rate and the extent of uptake [50]. A third issue related to brain uptake is the intrabrain distribution, i.e. the distribution of drug or PET radiotracer to intra- and extracellular areas of the brain, including the hydrophilic and lipophilic micro-environments and the intracellular brain organelles. Further, a fraction of the drug may be specifically bound to targets, e.g. receptors or enzymes.

Rate of brain uptake

The transport rate across the BBB depends on the physicochemical properties of the molecule, such as lipophilicity which governs passive diffusion, the existence of active uptake mechanisms and the involvement of active efflux. The rate is the net sum of inward and outward transport, i.e. transport rate is decreased by efflux transporters and increased by active influx and
can be estimated using the permeability surface area product (PS), clearance (CL) or rate constant (k).

Extent of brain uptake
The extent of brain uptake is related to the concentration or amount of the drug in the brain at a specific time point, often at steady-state. The amount of drug in the brain is often compared with the amount of drug in the plasma. The partition coefficient, \( K_p \), describes the ratio between the total concentration in the brain and the total concentration in plasma. However, many drug molecules are bound to plasma proteins and are therefore not available for transport across the BBB. To compensate for plasma protein binding, the partition coefficient based on the concentration of drug unbound to plasma proteins, \( K_{p,uu} \), describes the ratio between the total concentration in brain and the unbound concentration in plasma. Molecules also bind to brain tissue and, to account for this, \( K_{p,uu} \) is defined as the ratio between the concentration of unbound drug in brain interstitial fluid and the concentration of unbound drug in plasma at steady-state [51]. The concentrations of unbound drug in plasma and the brain will be equal when steady-state has been reached, if the drug is not being actively influxed into or effluxed out of the brain. Since the bulk flow is small (around 0.2 μL/min/g-brain [52]), most compounds will display a \( K_{p,uu} \) of 1 if they are unaffected by active efflux. If the ratio is less than unity at steady-state, it is likely that there is some type of active efflux at the BBB. Thus, the ratio of unbound drug concentrations in the brain to those in plasma, \( K_{p,uu} \), will describe the effectiveness of the active transport processes at the BBB.

Intrabrain distribution
The volume of distribution of unbound drug in the brain (\( V_{u,br} \) in mL/g-brain) is a useful measure of drug brain distribution, describing the relationship between total and unbound drug concentrations in the brain independently of BBB transport:

\[
V_{u,br} = \frac{A_{tot,br} - V_{bl} \cdot C_{bl}}{C_{u,br}}
\]  

(1)

\( A_{tot,br} \) is the total (unbound, bound, intracellular etc) amount of drug in the brain, \( V_{bl} \) is the volume of blood in the brain tissue, \( C_{bl} \) is the concentration of drug in the blood and \( C_{u,br} \) is the concentration of unbound drug in brain. The interstitial fluid (ISF) volume in the brain is 0.2 mL/g-brain [53] and this is therefore the smallest \( V_{u,br} \) possible, meaning that the drug is distrib-
uted mainly in the ISF. A value close to the brain water volume (0.8 mL/g-brain) indicates even distribution through the whole of the brain tissue. Larger $V_{u,br}$ values indicate that the drug has affinity for brain tissue. Hence, the drug distribution inside the brain can be understood by comparing $V_{u,br}$ with physiological volumes.

**Positron Emission Tomography**

PET is a functional imaging method that can be used for studying physiological, biochemical and pharmacological processes in various tissues *in vivo*. A PET radiotracer is a molecule labelled with a positron-emitting radionuclide, such as $^{11}$C, $^{13}$N, $^{15}$O, $^{18}$F or $^{68}$Ga, which binds to the biological target under investigation, for example a receptor, enzyme or transporter [54]. The radionuclide decay of $^{11}$C is illustrated in Figure 2. PET has been widely used to locate tumours in the brain and the rest of the body [55,56]. Because tumours overexpress uptake transporters for natural nutrients, they can be located using labelled analogues of glucose, amino acids or nucleosides. The recent development of dual modality scanners, i.e. PET combined with computerized tomography (CT) has enabled the generation of images displaying both the function and the anatomical structures of the tissue. This has been a major advance in medical imaging technology and has further established PET as an important tool in medical diagnostics and pharmaceutical research.

![Figure 2. Decay of $^{11}$C. A positron-emitting nuclide, in this figure $^{11}$C, is unstable due to a surplus of protons. Therefore, one proton is converted to a neutron to increase stability and, at the same time, a positron is emitted from the nucleus. The positron travels through tissue for up to a few mm until it collides with an electron. The positron-electron annihilation produces two photons that are emitted at an angle of approximately 180 degrees. These photons are then detected by the PET scanner.](image)
The radionuclides commonly used in PET have a relatively short half-life, 20.3 min for $^{11}$C and 109.8 min for $^{18}$F. Thus, the tracer synthesis time has to be short and the PET tracer must be injected into the study subject shortly after it has been synthesized. The PET investigation can be a static or dynamic operation. A static PET investigation has one time frame and results in one set of images for the whole investigation period. For a dynamic PET investigation the data is divided into a number of successive time frames, resulting in several sets of images over consecutive time frames. The sequence of PET images obtained during a dynamic PET investigation provides a quantitative estimation of the radiotracer concentration in the tissue over time. A region of interest (ROI) is often outlined on the PET image around the tissue or part of a tissue that is of interest for the study. The ROI can be applied to images from different time frames to generate a dynamic time-activity curve for the particular region.

Novel labelling methods have provided new opportunities for labelling functional groups to increase the number of possible radiotracers. Since most drug molecules contain carbon, there is interest in $^{11}$C labelling, where an isotopically unmodified carbon atom is replaced by $^{11}$C, thus not altering the molecule with respect to its pharmacokinetic properties. Most small drugs or drug-like compounds can now be labelled with $^{11}$C and PET has subsequently become an important tool in the development of new drugs, especially anti-psychotic and anti-depressive drugs [57-59]. PET has also been used for studying neurological disorders such as anxiety, epilepsy and Alzheimer’s and Parkinson’s diseases [60-63].

There are three principal methods by which PET can be used to understand drug behaviour in vivo:

- Using a labelled drug to understand its administration, distribution, metabolism or elimination (ADME)
- Using a labelled entity which allows imaging of the target and drug action on the target
- Using a labelled entity which reflects the effects of the drug on cellular or organ physiology.

In the first scenario, PET can be used to study the pharmacokinetics of the drug molecule directly, so that drug uptake in the brain, time to maximum concentrations in the brain, and brain concentrations over time can be obtained. Optimally, the labelled drug is co-injected with unlabelled drug and, since the relationship between labelled and unlabelled drug concentrations is known, the tissue pharmacokinetics can then be deduced quantitatively at clinically relevant doses. However, if the drug is only slowly distributed to the brain, the information obtained in this experimental setting might have
limited value since a PET investigation cannot be extended beyond 3-4 half-lives of the radionuclide.

In the second scenario, a radiotracer is used as a marker for specific target systems. In this case, it is changes in the distribution of the radiotracer after administration of the drug that is studied in the PET investigation. For example, if a radiotracer is known to bind to a specific receptor, a PET scan before drug administration allows a quantitative estimate of the tracer’s binding to this receptor. A new PET scan after drug challenge allows an estimate of the proportion of receptors that have been occupied by the drug. The time aspects of elimination of the drug from its brain targets can be obtained by performing multiple PET scans at different time points. This rate of reduced action on the receptor can then be related to the plasma pharmacokinetics and used for a refined definition of drug movements. Further, the receptor occupancy can be determined by measuring the amount of radiotracer in the brain after different doses of drug, thereby helping to determine the recommended dose. An issue that must be critically considered with PET is the cost and time it takes to develop a new method of labelling a new molecule. To use an already established radiotracer may therefore be an advantage.

Finally, a radiotracer might be used to monitor the effect of the drug on cellular function. For example, an $^{18}$F-labelled glucose analogue is frequently used for studying cancer tumours; in this case, a high uptake indicates extensive metabolism and a viable tumour, and decreased uptake after treatment suggests that treatment was successful.

In this thesis, PET has been used primarily to evaluate drug distribution to the brain using labelled drugs. PET can only measure total concentrations in the brain and hence only $K_p$ and $K_{p,u}$ can be calculated. If a drug is extensively bound to brain tissue, the ratios of total concentrations in brain to total or unbound concentrations in plasma can be larger than unity even if active efflux is present. Hence, the unbound ratio, $K_{p,uu}$, cannot be estimated from a PET investigation unless the unbound fraction in the brain is deduced by other means. In vitro methods for this purpose was recently presented by Fridén et al. [64]. Nonetheless, $K_p$ and $K_{p,u}$ in brain can be compared before and after modulation of BBB function. $K_p$ is often referred to as the tissue distribution volume, called $V_d$ in PET literature [65]. This PET nomenclature can be confusing, since the distribution volume in standard pharmacokinetics refers to the apparent volume of distribution for a drug, given in volume units. In this thesis, $K_p$ will be used to indicate the tissue-plasma partition ratio and $V$ will be used as a measure of distribution volume according to standard pharmacokinetic nomenclature.

Further, since PET measures total radioactivity, metabolites carrying the radioactive label will also contribute to the signal, thus potentially confounding the results. Hence, radiotracers need to be investigated with re-
spect to metabolism and the appearance of radioactive metabolites in plasma and in tissue, as well as with respect to their pharmacokinetics.

**Alternative methods of studying brain uptake**

Several *in vivo* or *in situ* methods of studying the transport of drugs into the brain have been published. Most of these include measurement of total drug concentrations in the brain shortly after drug administration.

The brain uptake index method was developed by Oldendorf [66]. The drug and a reference compound are injected into the carotid artery of the animal, which is decapitated 15 seconds later. The reference compound should be highly diffusible and flow-limited. The drug uptake is calculated by dividing the ratio of the drug and reference compound concentrations in brain by the ratio of the drug and reference compound concentrations in the injection solution.

In the intravenous injection technique, the drug is administered to a group of animals, either as a bolus or as a constant infusion [67]. The animals are decapitated at different time points and the drug concentrations in brain and plasma are measured. The transport rate into the brain is obtained by division of the total amount of drug in the brain tissue by the area under the plasma concentration-time curve. If active efflux exists, the obtained rate will be the net transport rate of drug into the brain, i.e. the transport rate into the brain minus the transport rate out of the brain due to active efflux.

In the *in situ* brain perfusion method, the brain is perfused by a solution containing the drug for up to 120 seconds [68]. The animal is decapitated when the perfusion is stopped and the amount of drug in the brain is determined. The transport rate into the brain is calculated by dividing the amount of drug in the brain by the drug concentration in the perfusate multiplied by the perfusion time. As in the intravenous injection technique, the transport rate is the net transport rate into the brain.

The brain efflux index method has been used to estimate drug efflux from the brain [69]. The drug and an impermeable reference compound are injected into the brain. The ratio of the amounts of drug and reference compound in the brain is divided by the ratio of the amounts of drug and reference compound that were injected.

All of the above methods are used only in preclinical settings, because they require brain tissue sampling. Further, only one measurement point can be obtained from each animal.

Microdialysis is an *in vivo* method that measures unbound drug concentrations over time [70-72]. A probe containing a semipermeable membrane is inserted into the tissue or fluid of interest and is continuously perfused with a physiological solution (the perfusate). The dialysate, i.e. the fluid leaving the probe, is collected in fractions. Depending on the concentration...
gradient, molecules will either have been delivered to or recovered from the area surrounding the probe. For example, a drug injected intravenously will be detected in the dialysate if it has been distributed to the tissue under investigation. The invasiveness of the microdialysis method limits its use in human subjects. Further, local tissue damage caused by insertion of the probe into the brain could confound the results in BBB transport studies. The method also requires tubing and since many substances adhere to the tubing materials the method has limitations regarding studies of lipophilic compounds.

Drug concentrations in the cerebrospinal fluid (CSF) are sometimes used as a substitute for concentrations in the brain. However, the CSF is separated from the blood by the blood-CSF barrier and the permeability properties of this membrane are different from those of the BBB. Because sampling of CSF in humans takes place far from the central CSF space, the local equilibrium between extracellular fluid and brain concentrations of drug may differ from the CSF-brain equilibrium, resulting in wide variations in drug concentrations between the two compartments [73-77]. CSF is sometimes obtained in humans but the sampling is limited since it is an invasive method.
Aims of the Thesis

The general objective of this thesis was to investigate in vivo the influence of active efflux transporters on the transport of molecules across the blood-brain barrier.

The specific aims were:

- To develop new PET methodologies for studies of active efflux at the blood-brain barrier;

- To apply pharmacokinetic modelling and simulations to investigate the mechanisms of active efflux and how it influences the brain pharmacokinetics of efflux transporter substrates;

- To investigate time-related and quantitative aspects of cyclosporin A-induced P-glycoprotein inhibition;

- To study species differences in blood-brain barrier transport with emphasis on P-glycoprotein.
Materials and Methods

Animal studies

Male Sprague-Dawley rats, weighing 300-600 g, were used in the studies described in paper II-V, Dunkin-Hartley guinea pigs (400-600 g) were used in paper V. The animals were housed at 20°C with a 12-hour light-dark cycle and free access to food and water at least 7 days before the experiments and were transported to Uppsala Imanet on the day before the experiments. The cynomolgus monkeys used in paper V (3.8 and 6.4 kg) were transported to Uppsala Imanet on the morning of the experiments. The Göttingen minipigs used in paper V (23-46 kg) were transported from the animal facilities to the PET facilities an hour before the experiments. All animal experiments were approved by the Uppsala Animal Ethics Committee (C117/4 and C 153/4) or the Danish Animal Research Inspectorate (Journal number 2003/561-745).

Human studies

The clinical data used in paper V were acquired from clinical studies carried out earlier in accordance with the Helsinki declaration [78,79]. The studies were approved by the local ethics and radiation safety committees. Written consent was obtained from all subjects.

Radiotracers used in the studies

Verapamil is a calcium-channel blocker used for treatment of hypertension, angina pectoris and cardiac arrhythmias. The labelled version of verapamil, i.e. \([^{11}C]\)verapamil, has been used mainly for studies of the P-gp function [19-22,80,23-27]. Verapamil was labelled with \(^{11}\text{C}\) using \([^{11}C]\)methyl iodide (paper II, III and V). The radiochemical purity was >95%. The synthesis took a total of 30 min from the end of the bombardment. The specific activity was >20 GBq/μmol.
Hydroxyurea inhibits DNA synthesis and is used clinically for treatment of sickle-cell anaemia, HIV, head and neck cancer, and chronic myeloid leukaemia [81-85]. Hydroxyurea was labelled with $^{11}$C using $[^{11}$C$]$carbon monoxide (paper IV). The radiochemical purity was >99%. The synthesis took less than 25 min and the compound was isolated with a radioactivity of approximately 500 MBq.

GR205171 is an NK$_1$ receptor antagonist which has been investigated as a drug for treatment of chemotherapy-induced nausea and social anxiety disorder [86,62]. $[^{11}$C$]$GR205171 was obtained by alkylation with $[^{11}$C$]$methyl iodide (paper V). The radiochemical purity was >98% and the specific activity was about 50 GBq/µmol. The synthesis took 45 min from the end of the radionuclide production.

Altanserin is an antagonist for the serotonin 5HT$_{2A}$ receptor. $[^{18}$F$]$altanserin has been used in studies of serotonergic function in both healthy subjects and patients suffering from various neurological diseases [87-89]. Altanserin was labelled with $^{18}$F using $[^{18}$F$]$fluoride (paper V). The radiochemical purity was >95% and the specific activity was >110 GBq/µmol. The preparation time was about 2 hours.

A more detailed description of the synthesis of $[^{11}$C$]$verapamil can be found in paper II and III. The synthesis of the other radiotracers has been described previously: $[^{11}$C$]$hydroxyurea by Barletta et al. [90], $[^{11}$C$]$GR205171 by Bergström et al. [91] and $[^{18}$F$]$altanserin by Lemaire et al. [92].

The chemical structures of the radiotracers are shown in Figure 3, where the asterisk represents the position of the radiolabel. In this thesis, the radiotracers were administered at sub-pharmacological doses and studied mainly with regard to their ability to penetrate the BBB.

![Chemical structures](image)

**Figure 3.** Chemical structures of the radiotracers discussed in this thesis. The asterisk represents the radiolabel position.
Experimental procedures

Study design

The substances studied in paper I-IV were administered by various methods. In paper I, a computer-simulated exponential infusion was stopped after 8 hours. In paper II and IV, the radiotracer was administered as an exponential stepwise infusion during the 2-hour PET investigation and the transporter inhibitors were administered as fast bolus injections 30 min after the start of the administration of radiotracer. In paper III, both radiotracer and inhibitor were administered as infusions. The radiotracer was infused over 70 min and the inhibitor was administered over 100 min, starting 20 min after radiotracer administration. The experimental design used in paper III is illustrated in Figure 4. In paper V, the radiotracer was administered as a fast bolus and the inhibitor was administered as a bolus plus infusion. Administration of the inhibitor was started 20-25 min prior to starting the radiotracer and was continued during the 60-min PET investigation. The exponential and bolus-plus-infusion modes aimed to maintain constant plasma concentrations. A summary of the experimental designs is provided in Table 2.

Figure 4. Experimental design of the PET study in Paper III. $[^{11}\text{C}]$verapamil was administered as an exponential stepwise infusion via a computerized pump system to obtain steady-state concentrations in the animal. The inhibitor drug, cyclosporin A, was administered as a bolus followed by a constant infusion started after steady-state concentrations of $[^{11}\text{C}]$verapamil had been attained and continued after the $[^{11}\text{C}]$verapamil infusions were stopped. The uptake of $[^{11}\text{C}]$verapamil was investigated dynamically in a sequence of PET scans in parallel with measurement of $[^{11}\text{C}]$verapamil (V) and CsA concentrations in blood.
Table 2. Experimental design and administration regimens for radiotracers and inhibitors used in paper I-V

<table>
<thead>
<tr>
<th>Paper</th>
<th>Radio-tracer/marker administration mode</th>
<th>Radiotracers</th>
<th>Inhibitor administration mode</th>
<th>Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>exponential infusion</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>II</td>
<td>exponential infusion</td>
<td>$[^{11}C]$verapamil bolus injection</td>
<td>cyclosporin A: 3, 10 and 25 (mg/kg)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>exponential infusion</td>
<td>$[^{11}C]$verapamil bolus plus infusion</td>
<td>cyclosporin A: 7.5 + 2.5, 15 + 5, 22.5 + 7.5 and 45 + 15 (mg/kg + mg/kg/h)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>exponential infusion</td>
<td>$[^{11}C]$hydroxyurea $[^{11}C]$verapamil bolus injection</td>
<td>probenecid: 50 and 150; hydroxyurea: 50, 150 and 450; cyclosporin A: 25 (mg/kg)</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>bolus injection</td>
<td>$[^{11}C]$verapamil $[^{18}F]$altanserin bolus plus infusion</td>
<td>cyclosporin A: 22.5 + 7.5 (mg/kg + mg/kg/h)</td>
<td></td>
</tr>
</tbody>
</table>

NA Not applicable in the simulation study.

A single experiment with $[^{11}C]$GR205171 was performed using the same protocol used for the $[^{11}C]$verapamil studies presented in paper III. In this study, the highest dose of CsA, 45 mg/kg followed by a constant infusion of 15 mg/kg/h, was administered to the animal.

Time-activity curves were obtained by outlining ROIs in the cortical and central structures of the brain (paper II-IV). The ROIs were summed up to a volume-of-interest. In paper V, ROIs were delineated in the region of the cerebellum where the distribution of NK₁ and 5HT₂A receptors is negligible [93,91,94]. In the monkeys administered $[^{11}C]$GR205171, ROIs were also drawn over the striatum where NK₁ receptors are abundant. In the minipigs administered $[^{18}F]$altanserin, ROIs were also drawn over the 5HT₂A receptor-rich region in the frontal cortex.

In $[^{18}F]$altanserin rat studies (paper V), the brain was dissected at 60 min and the radioactivity was measured in plasma, cerebellum and frontal cortex.
Blood samples were obtained in parallel with the PET scans and the whole blood and plasma levels of radioactivity were measured in a well counter that was cross-calibrated with the PET system. Additional blood samples for CsA analysis were obtained in paper III and V. In paper II, a parallel group of animals was used to study CsA concentrations in blood. The CsA concentration analysis was performed using immunoenzymatic assays: EMIT on a Cobas MIRA analyzer (Behring Diagnostic, France, paper II and III) and CEDIA (Microgenics, Ekerö, Sweden, paper V), both methods with a limit of detection of 25 ng/mL.

Animal surgery
Prior to each experiment, the rodents were placed in a Plexiglas container and anaesthetized with 3.6% isoflurane. They were kept anaesthetized with 2.2-2.6% isoflurane administered via a mask during the whole experiment. In paper V, the rats administered with [18F]altanserin were initially anaesthetized with an intravenous injection of 1 mL hyponorm/midazolam solution followed by 0.5 mL every 30 min. A catheter with 10 IE heparin in saline to prevent clotting was inserted into the left femoral artery to obtain arterial blood samples. An intravenous catheter was inserted into the left femoral vein of guinea pigs and into the tail vein of rats for radiotracer and CsA administration.

The minipigs used for the [18F]altanserin studies in paper V were initially anaesthetized with 0.15 mL/kg of intramuscular midazolam and Zoletil 50 vet. The anaesthesia was maintained with intravenous propofol (1 mL/kg/h). The minipigs were intubated endotracheally and ventilated during the entire experiment. During the scan, they were placed on a heating carpet and covered to maintain body temperature. Catheters were surgically inserted in the femoral vein for radiotracer and CsA administration, and into the femoral artery for blood sampling.

The cynomolgus monkeys (paper V) were lightly sedated with ketamine before venous catheters were inserted in both hind legs and propofol was administered to induce anaesthesia. The catheters were also used for administration of the radiotracer and Ringer-acetate. The animals were kept anaesthetised with 1.3-2.5% sevoflurane via tracheal intubation during the PET scan. A venous catheter inserted in the tail was used for blood sampling.

PET scanners
A microPET R4 scanner (Concorde Microsystems, Knoxville, TN, USA) was used in paper II-IV, and for the [11C]GR205171 and [11C]verapamil rodent studies in paper V. A combined PET/CT Discovery LS scanner (General Electrics, Milwaukee, WI, USA) was used for the [18F]altanserin
studies in minipigs and a Hamamatsu SHR-7700 PET scanner (Hamamatsu Photonics, Hamamatsu, Japan) was used for the \([^{11}\text{C}]\text{GR205171}\) and \([^{11}\text{C}]\text{verapamil}\) studies in monkeys (paper V). The human \([^{11}\text{C}]\text{GR205171}\) studies were performed using an ECAT EXACT HR+ scanner (Siemens/CTI, Knoxville, TN, USA) and the human \([^{18}\text{F}]\text{altanserin}\) studies were performed using a GE Advance scanner (General Electrics, Milwaukee, WI, USA) (paper V). Transmission scans were run prior to the emission scans to correct for attenuation.

### Metabolite analysis

The blood was centrifuged at 3000 x g for 2 min at 4°C (Beckman Allegra X-22R Centrifuge, Palo Alto, CA, USA) to separate whole blood from plasma. The plasma proteins were precipitated with acetonitrile (1:1) and centrifuged at 20800 x g at 4 °C for 1 min (Eppendorf 5417R centrifuge, Eppendorf AG, Hamburg, Germany). The supernatant was filtered through a 0.2 μm microcentrifuge filter (Costar Spin-X, Corning incorporated, Corning, NY, USA).

The brain sample was weighed, acetonitrile (1 mL per 1 g tissue) was added and the sample was then homogenized with a Polytron homogenizer PT 3000 (Kinematica, Littau, Switzerland). The homogenate was centrifuged at 3200 x g for 2 min at 4°C (Beckman Spinchron Centrifuge, Palo Alto, CA, USA) and the supernatant was filtered through a 0.2 μm microcentrifuge filter (Costar Spin-X, Corning incorporated, Corning, NY, USA).

The filtrates were injected together with isotopically unmodified reference compounds onto a Genesis C18 250 x 10 mm column (Grace Vydac, Hesperia, CA, USA). The intact radiotracer was eluted in the last fraction while the more polar metabolites were eluted in earlier fractions. Radioactivity was measured in all fractions and the percentage of intact radiotracer was calculated as:

\[
\text{Intact radiotracer} = \frac{\text{Radiotracer fraction}}{\text{All fractions}} \cdot 100
\]  

### Plasma protein binding

The plasma protein binding of the radiotracers was determined by equilibrium dialysis in paper V. Plasma samples were obtained prior to tracer injection, 1-2 MBq of the radiotracer was added and 500 μL of the sample was then transferred to a dialysis chamber with a semipermeable membrane separating it from another chamber containing 500 μL of PBS buffer. In contrast to protein-bound molecules, unbound radiotracer molecules freely diffuse across the membrane. The chambers were shaken in a water bath at
37°C for 2 hours, before a sample from each chamber was removed and the radioactivity concentration was measured. The fraction of radiotracer unbound to plasma proteins, $f_p$, was calculated according to Equation 3:

$$f_p = \frac{R_{\text{buffer}}}{R_{\text{plasma}}} \cdot 100 \quad (3)$$

where $R_{\text{buffer}}$ and $R_{\text{plasma}}$ are the radioactivity concentrations in the buffer and plasma chambers, respectively.

**Data analysis**

**Simulations**

In paper I, a computerized three-compartment brain model was used to simulate drug pharmacokinetics in the brain under the influence of different types of active efflux. The concentration-time profiles in the three compartments were simulated using MATLAB 6.5 (Mathworks, Natick, MA, USA). The model consisted of passive processes in both the luminal and abluminal membranes of the endothelial cells, in combination with efflux pumps located at both membranes of the BBB and is shown in Figure 5.

**Figure 5.** The model used in the simulation study. Single compartments were used for the blood, the endothelial cells of the blood-brain barrier and the brain. Passive transport ($CL_{\text{pass}}$) in two directions was present at both the luminal and abluminal membranes. Active transport according to the influx hindrance mechanism at the luminal membrane ($CL_1$) and the efflux enhancement mechanism at the luminal ($CL_2$) and abluminal ($CL_3$) membranes was present in the direction from brain to blood.
It was assumed that there was instant equilibrium between the concentration of drug in the blood and that in the luminal membrane. Similarly, the concentration in the abluminal membrane was assumed to equilibrate instantly with that in the brain ISF. Therefore, the luminal membrane drug concentration was assumed to be equal to the concentration in blood, and the concentration in the abluminal membrane was assumed to be equal to the concentration in the brain ISF. Passive clearance was assumed to occur at the same rate across the luminal and abluminal membranes. The concentrations of unbound drug in blood (\(C_{u,bl}\)), endothelium (\(C_{u,ec}\)) and brain ISF (\(C_{u,br}\)) were described by Equations 4-6. Single compartments were used for the blood, the cytosol of the endothelial cell, and the brain. Although this is a simplification for most compounds, it clarified the consequences of the processes at the BBB on brain ISF concentration-time profiles.

\[
V_{u,bl} \frac{dC_{u,bl}}{dt} = R_0 - (CL + CL_{pass} - CL_1) \cdot C_{u,bl} + (CL_{pass} + CL_2) \cdot C_{u,ec} \tag{4}
\]

\[
V_{u,ec} \frac{dC_{u,ec}}{dt} = (CL_{pass} - CL_1) \cdot C_{u,bl} - (2 \cdot CL_{pass} + CL_2) \cdot C_{u,ec} + (CL_{pass} + CL_3) \cdot C_{u,br} \tag{5}
\]

\[
V_{u,br} \frac{dC_{u,br}}{dt} = CL_{pass} \cdot C_{u,ec} - (CL_{pass} + CL_3) \cdot C_{u,br} \tag{6}
\]

\(V_{u,bl}\), \(V_{u,ec}\) and \(V_{u,br}\) are the volumes of distribution of unbound drug in blood, endothelium and brain, respectively. The infusion rate is described by \(R_0\). In Equations 4–6, \(CL\) is the unbound systemic clearance, \(CL_{pass}\) is the unbound passive clearance across the BBB, and \(CL_1\) is the clearance by influx hindrance, i.e. the process hindering the drug from reaching even the cytosol of the BBB endothelial cells as it is transported back to the blood. \(CL_2\) is the clearance by efflux enhancement at the luminal membrane, i.e. transporting the drug from the cytosol of the endothelial cell to the blood. \(CL_3\) is the clearance by efflux enhancement at the abluminal membrane, i.e. transporting the drug from the brain ISF to the cytosol of the BBB endothelial cell.

The rat was used as a model, since experimental data for this species is available for comparison with the simulations. In the first set of simulations, it was assumed that the compounds were uniformly distributed throughout the body, and the physiological volumes of total body water (167 mL) and brain (0.7 mL/g-brain) in rats were used as volumes of distribution in the blood and brain compartments, respectively [95]. A value of 0.8 µL/g-brain was assigned as the volume of brain endothelial cells [96,97].

The body clearance (CL) was set at 5 mL/min, resulting in an elimination constant (\(k_e\)) out of the body of 0.03/min and a half-life of 23 min. The con-
The concentration of unbound drug in the blood was set at the steady-state value ($C_{u,ss,bl}$) at time 0 to clarify the time for equilibration across the BBB. The infusion rate ($R_0$) was equal to $C_{u,ss,bl} \cdot CL$ to maintain steady-state concentrations with an infusion time of 8 hrs. The simulations were continued for a further 8 hrs following the end of the infusion. These relatively long durations were used so as to follow the concentrations of poorly permeating model compounds. $CL_{pass}$ values of 0.3, 3, 10, 30, and 300 µL/min/g-brain were used in the simulations. This range was based on the reported permeability clearances of a set of opiates [98-100,47]. Active efflux clearances, $CL_1$, $CL_2$ and $CL_3$, were set at 5 or 10 µL/min/g-brain.

The influence of the brain volume of distribution on the brain concentration profiles was investigated in a second set of simulations in which the other parameters were kept constant. The $V_{u,br}$ was first set at 0.2 mL/g-brain, aiming to describe distribution solely into the brain ISF. Increasing $V_{u,br}$ values to 2, 5, and 10 mL/g-brain were then studied in the simulations.

Modelling of the $[^{11}C]$verapamil and cyclosporin A interaction

The influence of CsA on P-gp-mediated active efflux of $[^{11}C]$verapamil from brain to blood was modelled with MATLAB 7.0 (Mathworks, Natick, MA, USA) (paper II) and NONMEM VIβ (GloboMax LLC, Hanover, MA, USA) (paper III). In paper III, pharmacokinetic models for CsA and $[^{11}C]$verapamil in blood were developed and then included in the final model describing the transport of $[^{11}C]$verapamil across the BBB. In paper II, no blood samples were obtained during the PET scans but the plasma concentration of $[^{11}C]$verapamil was kept constant during the scan (verified in separate studies). Hence, the transport rate of $[^{11}C]$verapamil into and out of the brain at steady-state was assumed to be constant and no value for $k_{in}$ was obtained in this paper.

\[ k_{in} \cdot VER_{pl,ss} = k_{out} \cdot VER_{br,ss} \]  

(7)

where $k_{in}$ and $k_{out}$ are the rate constants for the inward and outward transport of $[^{11}C]$verapamil, respectively, and $VER_{pl,ss}$ and $VER_{br,ss}$ are the steady-state concentrations in plasma and brain, respectively.

The administration of CsA either increases transport of the marker, $[^{11}C]$verapamil, into the brain or decreases its transport out of the brain [101]. If CsA modulates the transport of verapamil into the brain, i.e. increases $k_{in}$, the concentration of $[^{11}C]$verapamil in the brain after CsA administration can be described by the equation:

\[ \frac{dVER_{br}}{dt} = E_{kin} \cdot k_{in} \cdot VER_{pl} - k_{out} \cdot VER_{br} \]  

(8)
where $E_{\text{kin}}$ is the effect of CsA on $k_{\text{in}}$ and is larger than or equal to 1. If, however, CsA modulates [$^{11}$C]verapamil transport out of the brain, i.e. decreases $k_{\text{out}}$, the [$^{11}$C]verapamil brain concentration can be described as:

$$\frac{d\text{VER}_{br}}{dt} = k_{\text{in}} \cdot \text{VER}_{pl} - E_{\text{kout}} \cdot k_{\text{out}} \cdot \text{VER}_{br}$$ \hspace{1cm} (9)$$

where $E_{\text{kout}}$ is the effect of CsA on $k_{\text{out}}$ and is a value between 0 and 1.

Indirect response models were used to describe the effect of CsA on the rate constants ($k_{\text{in}}$ or $k_{\text{out}}$) \cite{102}. 

$$E_{\text{kin}} = 1 + \frac{E_{\text{max}} \cdot \text{CsA}_{bl}}{IC_{50} + \text{CsA}_{bl}}$$ \hspace{1cm} (10)$$

$$E_{\text{kout}} = 1 - \frac{E_{\text{max}} \cdot \text{CsA}_{bl}}{IC_{50} + \text{CsA}_{bl}}$$ \hspace{1cm} (11)$$

$E_{\text{max}}$ denotes the maximum change in $k_{\text{in}}$ or $k_{\text{out}}$ after CsA administration, $IC_{50}$ is the CsA concentration required to achieve half the maximum change in $k_{\text{in}}$ or $k_{\text{out}}$, $\gamma$ is the sigmoidicity factor of the indirect response model and CsA$_{bl}$ is the concentration of CsA in blood. When CsA$_{bl}$ is zero, $E_{\text{kin}}$ and $E_{\text{kout}}$ will be 1, i.e. $k_{\text{in}}$ or $k_{\text{out}}$ are at their baseline values.

In paper II, a global minimization was performed, i.e. the model was fitted to all individuals at the same time and typical parameter values for [$^{11}$C]verapamil transport across the BBB and for the CsA effect model were obtained.

In paper III, mixed effects modelling was used. The fixed effects characterize the typical animal and the random effects include inter-animal and residual variability. The first order conditional estimation method with interaction (FOCE inter) was used throughout the modelling. The inter-animal variation was described by the exponential variance model:

$$P_i = P_{\text{pop}} \cdot \exp(\eta_i)$$ \hspace{1cm} (12)$$

where $P_i$ is the parameter in the $i^{th}$ animal, $P_{\text{pop}}$ is the parameter in the typical animal and $\eta_i$ is the inter-animal variability, assumed to be normally distributed around zero and with a standard deviation, $\omega$, to distinguish the $i^{th}$ animal’s parameter from the typical value as predicted from the regression model. The need for inclusion of inter-animal variability was investigated for all parameters. Additive and proportional error models were considered for the residual variability.
Non-compartmental analysis

The time-activity profiles in the brain and plasma were expressed as Standardized Uptake Values (SUV) to enable comparison between scans (paper IV and V).

\[
SUV = \frac{\text{Measured radioactivity in tissue or plasma}}{\text{Injected radioactivity} / \text{Body weight}}
\]  

(13)

The brain-to-plasma ratio, \(K_p\), was calculated using the SUV in plasma and brain.

\[
K_p = \frac{SUV \text{ brain region}}{SUV \text{ plasma}}
\]  

(14)

SUV in brain and plasma were measured and \(K_p\) was calculated over the scanning time. The effect of P-gp inhibition was expressed as ratios of SUV or \(K_p\) before and after CsA administration (paper IV) or in animals receiving CsA versus untreated controls (paper V).

\[
SUV_{\text{increase}} = \frac{SUV \text{ with CsA}}{SUV \text{ baseline}}
\]  

(15)

\[
K_{p,\text{increase}} = \frac{K_p \text{ with CsA}}{K_p \text{ baseline}}
\]  

(16)

Statistics

Model comparisons were based on the Akaike information criterion (AIC) [103] and the objective function value (OFV) in paper II and III, respectively. The model with the lowest AIC or OFV was considered the best. For nested models, an OFV drop of 3.83, 6.63 and 10.83 units corresponds to an improved fit at \(P < 0.05\), \(P < 0.01\) and \(P < 0.001\), respectively.

In all non-compartmental analyses, parameter values are reported as geometric means with the standard deviation or relative standard error. In paper V, the one-tailed t-test was used to determine whether the increase in SUV and \(K_p\) in CsA-treated animals compared to controls was statistically significant, while the two-tailed t-test was used to determine whether the plasma protein binding of the studied radiotracers was different in CsA-treated and control animals.
Results and Discussion

Comparison of different types of active efflux

The objective of the simulation study (paper I) was to investigate how the nature, location and capacity of the efflux processes in relation to the permeability properties influence time to reach steady-state concentrations in the brain (i.e. rate) and the brain concentrations at steady-state (i.e. extent). The time to reach steady-state and the half-life in the brain with no active efflux present is governed by the rate of passive transport across the BBB. At high passive transport rates typical for lipophilic drugs, steady-state is reached fast and the brain concentration-time profiles are almost parallel to the blood concentration-time profiles; hence, the half-life is the same in blood and brain. The same half-life in blood and brain is also observed, even if active efflux is present, when plasma clearance is lower than the clearance from brain to plasma, as was seen with $[^{11}\text{C}]$verapamil.

With lower passive clearance, the time to reach steady-state and the half-life in the brain is also extended because of slower redistribution between brain and blood. However, the same steady-state unbound drug concentration in blood and brain is reached, i.e. $K_{puu}$ is 1, independently of the rate of passive clearance. This is illustrated in Figure 6a and Table 3.

The simulations showed that efflux enhancement decreased the drug half-life in the brain compared with passive transport alone, while influx hindrance had no effect on the half-life in the brain. Drug already positioned in the endothelial cells or in the brain is not picked up by an influx hindrance transporter, and influx hindrance can therefore not reduce the brain half-life. Efflux enhancement, on the other hand, takes molecules that are inside the endothelial cells or the brain and extrudes them, and therefore this mechanism will affect the half-life. In theory, if BBB transport is the rate-limiting step, the half-life at different degrees of inhibition can give an indication of which of the two mechanisms an efflux transporter is using to extrude its substrates. However, the half-life in brain for $[^{11}\text{C}]$verapamil is rate-limited by the elimination of $[^{11}\text{C}]$verapamil from the blood, i.e. elimination from the blood is slower than transport between the brain and the blood. Thus, the half-life in blood is the rate-limiting step for brain elimination of $[^{11}\text{C}]$verapamil. The half-life of $[^{11}\text{C}]$verapamil in plasma and brain was
about 50 min at all blood concentrations of CsA (paper III). Consequently, no conclusions could be drawn based solely on the half-life in the brain about whether CsA was affecting [\textsuperscript{11}C]verapamil transport into or out of the brain.

The simulations showed that an influx hindrance mechanism at the luminal membrane (CL\textsubscript{1}) was the most effective mechanism for lowering brain concentrations, Figure 6b. It resulted in the lowest unbound brain to blood concentration ratios, Table 3. Efflux enhancement on the abluminal side of the BBB resulted in a K\textsubscript{p,uu} equal to that for the same process on the luminal side, but the time to steady-state and the half-life in the brain were shorter than those in the luminal model.

### Table 3. Brain-to-blood ratios of unbound drug concentrations (K\textsubscript{p,uu}) and half-lives in brain at different clearance types. The active clearances (CL\textsubscript{1}, CL\textsubscript{2} and CL\textsubscript{3}) were set at 10 μL/min/g-brain, CL was set at 5 mL/min, K\textsubscript{t} at 1000 ng/mL, V\textsubscript{u,bl} at 167 mL, V\textsubscript{u,ec} at 0.8 μL/g-brain and V\textsubscript{u,br} at 0.7 mL/g-brain

<table>
<thead>
<tr>
<th>CL\textsubscript{pass} \textsuperscript{a}</th>
<th>No active transport</th>
<th>CL\textsubscript{1} \textsuperscript{b}</th>
<th>CL\textsubscript{2} \textsuperscript{b}</th>
<th>CL\textsubscript{1}&amp;CL\textsubscript{2} \textsuperscript{b}</th>
<th>CL\textsubscript{3} \textsuperscript{b}</th>
<th>CL\textsubscript{2}&amp;CL\textsubscript{3} \textsuperscript{b}</th>
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<tr>
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<table>
<thead>
<tr>
<th>CL\textsubscript{pass} \textsuperscript{a}</th>
<th>Brain-to-blood unbound concentration ratio (K\textsubscript{p,uu})</th>
<th>Half-life in brain (min)</th>
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</thead>
<tbody>
<tr>
<td>0.3</td>
<td>3255</td>
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<td>3</td>
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<td>200</td>
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<td>35</td>
<td>35</td>
</tr>
<tr>
<td>300</td>
<td>23</td>
<td>23</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Passive clearance in μL/min/g-brain.

\textsuperscript{b}Maximum active efflux (J\textsubscript{max}/K\textsubscript{t}) in μL/min/g-brain. At steady-state, the active clearances are somewhat lower, as the Michaelis-Menten ratio J\textsubscript{max}/(K\textsubscript{t} + C\textsubscript{u}) includes the concentration that drives the efflux pumps.

By varying the distribution volume of unbound drug in the brain, it was shown that a drug with a small volume of distribution will have a shorter half-life in the brain than a drug with a large volume of distribution. The K\textsubscript{p,uu} is not dependent on the volume of distribution, but the time to reach steady-state is longer for a drug with a large distribution volume.

The influence of the active processes on the brain time-concentration profiles decreases with increasing lipophilicity, given the same active process capacity.
a. The effect of various values of passive transport on the brain concentration-time profiles when no active transport mechanism is present. When given as an infusion, a compound with a lower passive clearance will take longer to reach steady-state than a compound with a higher passive clearance. However, they will both have the same unbound concentration in blood and brain interstitial fluid when steady-state is eventually reached (steady-state was not reached during the infusion time for the two lowest passive clearance values). A lower passive clearance will also result in a longer half-life in the brain [104].

b. The effect of active transport at the luminal membrane on brain concentration-time profiles. Influx hindrance (CL1) is more effective in decreasing drug concentrations in the brain than efflux enhancement (CL2) at this membrane. The combination of both active processes (CL1 and CL2) results in a brain-to-blood ratio of unbound drug concentrations similar to the product of the ratios for the two separate processes. Efflux enhancement results in a shorter half-life in the brain than that seen with passive transport alone, while influx hindrance does not affect the half-life in the brain. The thin lines describe the unbound drug concentration in blood with respective type of active transport.

Interaction of [11C]verapamil and cyclosporin A

[11C]verapamil is the most commonly used radiotracer for studies of P-gp function with PET [19-27]. [11C]verapamil has been administered as a single bolus in all of these studies. The drawback of this study design is the varying [11C]verapamil concentration during the PET scan. A common study design is to compare animals treated with a P-gp inhibitor (e.g. CsA) with untreated animals. If a single bolus injection of [11C]verapamil is used, it will be difficult to determine whether the inhibitor has affected the brain concentrations of [11C]verapamil by interaction with P-gp or by influencing the elimination or metabolism of [11C]verapamil. The same is true when the P-gp function in different patient groups is compared. Hence, it will be difficult to know if differences in [11C]verapamil brain concentrations are due to [11C]verapamil brain pharmacokinetics or to P-gp function. The impact of an intervention drug or a disease state on the P-gp system may therefore be masked by differences in the elimination or metabolism of [11C]verapamil.
If a bolus design is utilized, it is crucial that the pharmacokinetics in plasma is studied in parallel with the brain pharmacokinetics.

To avoid the drawbacks associated with a single bolus design, we chose to administer $[^{11}\text{C}]$verapamil as an exponential stepwise infusion to obtain steady-state concentrations in both brain and plasma. The $[^{11}\text{C}]$verapamil infusion rates were based on the brain kinetics observed after a single bolus injection of $[^{11}\text{C}]$verapamil. The elimination of $[^{11}\text{C}]$verapamil from the brain after a single bolus was best described by a triphasic elimination curve (half-lives: 0.22 min, 7.3 min and 103 min for the three phases, respectively). The exponential stepwise decreasing infusion rates over time were obtained by deconvolution of two vectors representing the desired curve, i.e. a steady-state concentration curve, and the brain concentration curve after a single bolus. The first short infusion was administered at the start of the emission scan and was followed by discrete short infusions at 15 s intervals during the first 10 min, at 30 s intervals between 10 min and 1 hour and at 1 min intervals between 1 hour and the end of the emission scan at 2 hours. Steady-state in the brain was obtained within minutes.

![Figure 7](image.png)

**Figure 7.** Uptake of $[^{11}\text{C}]$verapamil in brain with time. CsA (3 mg/kg open triangles, n=3, 10 mg/kg filled squares, n=3, 25 mg/kg open squares, n=4) was administered 30 min after start of the emission scan at steady-state concentrations of $[^{11}\text{C}]$verapamil as a short intravenous bolus dose. The study design enabled investigation of both the onset and the decline of P-gp inhibition. Each point represents the mean ± standard deviation.

In the study presented in paper II, CsA was administered as a bolus 30 min after the start of the PET scan. The inhibition of P-gp by CsA was a rapid process, both in the sense that P-gp was blocked immediately when CsA was administered and also in the sense that this inhibition was rapidly reversible when the CsA concentration decreased, Figure 7. When CsA was infused (paper III) instead of administered as a single bolus, steady-state
concentrations of CsA in blood were obtained around 25 min after initiation. This protocol was chosen so that a specific CsA concentration could be related to a certain degree of P-gp inhibition. The maximal increases in brain [11C]verapamil concentrations after CsA administration were 1.3-, 3.6-, 6.3- and 8.6-fold for the four CsA dose regimens. PET images of [11C]verapamil in the rat brain before and after CsA administration are shown in Figure 8. Both studies (paper II and III) showed that the brain and plasma concentrations of [11C]verapamil quickly reached equilibrium. Therefore, it was concluded that [11C]verapamil was a good substrate for P-gp interaction studies in the brain using PET, since changes in P-gp function can be detected as soon as they occur. This study also demonstrated the advantage of using steady-state concentrations of [11C]verapamil compared to a single bolus protocol, as each animal acted as its own control and the onset of the inhibition could be investigated.

Figure 8. PET images of [11C]verapamil before and after administration of CsA 45 mg/kg followed by 15 mg/kg/h. Brain [11C]verapamil concentrations increased 8.6-fold after CsA administration.

The metabolism of [11C]verapamil was not affected when CsA was administered as a bolus (paper II). However, when CsA was administered as a bolus followed by a constant infusion, the fraction of intact [11C]verapamil over time changed, both in the plasma and in the brain, as shown in Table 4. In plasma, the metabolite concentrations increased with increasing CsA concentration. This could have been due to CsA acting on P-gp in the renal proximal tubule causing a slower systemic elimination of metabolites [105,106]. In the brain, concentrations of both intact [11C]verapamil and the [11C]metabolite increased. The enhanced metabolite concentrations in the brain can be explained by similar increases in plasma metabolite concentrations, while the enhanced concentrations of intact [11C]verapamil were probably due to P-gp inhibition. Since the increase in [11C]verapamil concentrations was much larger than the increase in metabolite concentrations,
the fraction of intact $[^{11}\text{C}]$verapamil in the brain increased as CsA concentrations increased. In summary, the effect of CsA differed in brain and plasma: the fraction of intact $[^{11}\text{C}]$verapamil decreased in plasma and increased in the brain. Hence, it is important to study the metabolism of the radiotracer both at baseline and in the challenged states.

Table 4. Fraction (%) of intact $[^{11}\text{C}]$verapamil. Each value corresponds to one animal

<table>
<thead>
<tr>
<th>Time</th>
<th>Saline</th>
<th>$15\text{ mg/kg} + 5\text{ mg/kg/h CsA}$</th>
<th>$45\text{ mg/kg} + 15\text{ mg/kg/h CsA}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>45 min</td>
<td>70 min</td>
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<tr>
<td><strong>Plasma</strong></td>
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<td>59</td>
<td>37</td>
</tr>
<tr>
<td>70 min</td>
<td>59</td>
<td>59</td>
<td>32</td>
</tr>
<tr>
<td>95 min</td>
<td>42</td>
<td>44</td>
<td>19</td>
</tr>
<tr>
<td>120 min</td>
<td>34</td>
<td>36</td>
<td>18</td>
</tr>
<tr>
<td><strong>Brain</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>70 min</td>
<td>69</td>
<td>94</td>
<td>89</td>
</tr>
<tr>
<td>120 min</td>
<td>38</td>
<td>92</td>
<td>89</td>
</tr>
</tbody>
</table>

*Measurements at 45 and 70 min in each column are from the same animal and at 95 and 120 min from another animal.

The pharmacokinetic models indicated that CsA altered an efflux enhancement mechanism (paper II and III). The effect of CsA on P-gp inhibition is therefore likely to involve reduced transport of $[^{11}\text{C}]$verapamil out of the brain rather than increased inward transport. Since P-gp is situated at the luminal membrane, it is likely that the substrate molecules are taken up in the endothelial cells, i.e. before they have entered the brain, and then transported back to the plasma. It has been suggested that P-gp might extrude its substrates through both influx hindrance and efflux enhancement [10,12,6]. It may seem less rational that P-gp would work through efflux enhancement when influx hindrance seems to be more effective in keeping molecules out of the brain. However, molecules that have already passed across the BBB cannot be extruded by influx hindrance. An efflux enhancement mechanism on the luminal membrane is perhaps the “safest” option for a transporter that needs to protect the brain. If the transporter worked exclusively through influx hindrance, it could not efflux molecules that escaped the influx hindrance mechanism and entered the brain.

In paper II, inclusion of an effect compartment for CsA, possibly representing the endothelial cells of the BBB, resulted in the best fit. The AIC was, however, similar for models with or without an effect compartment, indicating a very small difference in fit between the two models. In paper III, CsA was administered as a bolus plus infusion instead of as a single bolus. The nine animals that were given single bolus injections of CsA (i.e. the data presented in paper II) were also included in the model. A two-
compartment model gave a good description of the CsA blood concentrations obtained both after infusion and after a single bolus.

![Diagram](image)

**Figure 9.** A schematic view of the full pharmacokinetic model of the verapamil-cyclosporin A interaction. CsA_{bl,c} and CsA_{bl,p} are the cyclosporin A concentrations in the central and peripheral blood compartments, and VER_{pl,c} and VER_{pl,p} are the [11C]verapamil concentrations in the central and peripheral plasma compartments, respectively. VER_{br,c} and VER_{br,p} are the [11C]verapamil concentrations in the central and secondary brain compartments, respectively. k_{10} and k_{50} are the systemic elimination rate constants for [11C]verapamil and cyclosporin A, respectively. The rate constants k_{12} and k_{21}, k_{34} and k_{43}, and k_{56} and k_{65} are the intercompartmental rate constants, k_{in} is the rate constant describing the transport of [11C]verapamil into the brain and k_{out} is the rate constant describing the transport of [11C]verapamil out of the brain.

The final pharmacokinetic model of the [11C]verapamil-CsA interaction at the BBB is presented in Figure 9 and the measured and predicted concentrations according to the model are shown in Figure 10. A two-compartment brain model gave the best fit. In contrast, a study by Lubberink et al., which
compared several one- and two-compartment brain models after a bolus injection of \( ^{11}\text{C}\)verapamil, indicated that a one-compartment brain model was sufficient for describing the brain kinetics of \( ^{11}\text{C}\)verapamil [107]. It is possible that if data from the elimination phase alone, i.e. after stopping the \( ^{11}\text{C}\)verapamil infusions, which resembles the situation after a bolus injection, had been modelled in our study, a one-compartment model might have been preferred, since the one- and two-compartment models differed very little during this phase. There may also be a species difference, as the study by Lubberink et al. was performed in human subjects.

**Figure 10.** Modelling results of the time-activity curves of \( ^{11}\text{C}\)verapamil in brain. Four different doses of CsA were administered at 20 min (n=16, 4 rats per CsA dose group). The verapamil infusions were stopped at 70 min. Each panel represents one animal; open circles represent measurements (DV), broken lines represent the population model predictions (PRED) and solid lines represent the model predictions for individual rats (IPRE). CsA doses: 7.5 mg/kg + 2.5 mg/kg/h (ID 1-4), 15 mg/kg + 5 mg/kg/h (ID 5-8), 22.5 mg/kg + 7.5 mg/kg/h (ID 9-12) and 45 mg/kg + 15 mg/kg/h (ID 13-16).
The IC50 of CsA, i.e. the concentration required to achieve 50% of full P-gp inhibition, was 8 μg/mL in paper II and 4.9 μg/mL in paper III. The difference might have been caused by the different CsA administration modes. The E_max values were 0.88 and 0.93, respectively, indicating that around 90% of the transport of [11C]verapamil from the brain to the blood was P-gp-mediated. This is a physiologically possible value, since it is unlikely that transport out of the brain would be completely inhibited (a situation corresponding to an E_max value of 1), as some passive transport across the BBB will always exist even if the P-gp function is completely inhibited. The sigmoidicity constant of the effect model, γ, was 2.1 and 2.5 in the respective studies.

The radiotracer [11C]verapamil is a racemic mixture of (R)-[11C]verapamil and (S)-[11C]verapamil. Since the S-enantiomer is metabolized faster than the R-enantiomer, it has been suggested that only the R-enantiomer should be used as a radiotracer for P-gp studies with PET [108,109]. Both enantiomers are transported to the same degree by P-gp and are therefore equally good markers for the P-gp function [110]. However, the use of the racemic mixture requires careful correction for metabolites and for longer studies it may be preferable to use the more slowly metabolized R-enantiomer.

Alternative radiotracers for active efflux studies

Beside [11C]verapamil, two other radiotracers, [11C]hydroxyurea and [11C]GR205171, were administered as exponential stepwise infusions. The brain pharmacokinetics of these two radiotracers was investigated after intervention with transporter inhibitors.

It was not possible to infuse [11C]hydroxyurea in such a manner that the brain concentrations were constant for most of the scan, since they kept increasing during the scan even after a single bolus dose. However, when the [11C]hydroxyurea concentrations in plasma were kept constant, the brain concentrations increased at a constant rate during the scanning period. It was assumed that if hydroxyurea was a substrate for an active efflux transporter, as indicated by Dogruel et al. [111], and this active transporter was inhibited, then the rate of increase in brain concentrations should be much faster than under baseline conditions. However, none of the possible inhibitors used (CsA, probenecid, unlabelled hydroxyurea) caused any change in the brain uptake of [11C]hydroxyurea (paper IV). In addition, unlabelled hydroxyurea, when used as an intervention drug in a [11C]verapamil PET study, did not increase brain uptake of [11C]verapamil. The high doses of probenecid and CsA, 150 mg/kg and 25 mg/kg, used in this study have been shown to be sufficient for inhibiting Mrp and P-gp, respectively, in the rat [98,100]. It is therefore highly unlikely that hydroxyurea is a substrate for or
an inhibitor of P-gp or a substrate for probenecid-sensitive transport systems. The low brain concentrations observed are likely to be due to a low uptake rate of the hydrophilic hydroxyurea. The pharmacokinetics of urea, a structurally related compound, further supports these findings. Urea is often used as a marker of BBB integrity [112-115]. It has been shown that, although urea penetrates into the brain, it does so slowly. In rats, at a constant plasma concentration, it takes up to 20 hours before plasma and brain urea concentrations are equal [116]. This study demonstrated the importance of separating the two concepts rate and extent of brain uptake.

![Graph](image)

**Figure 11.** Comparison of $[^{11}\text{C}]$verapamil (○, n=4, error bars = standard deviation) and $[^{11}\text{C}]$GR205171 (■, n=1) concentration increase after a CsA bolus of 45 mg/kg at 20 min followed by a constant infusion of 15 mg/kg/h. The maximum increase in brain concentration was of the same magnitude for both radiotracers: 8.6-fold for $[^{11}\text{C}]$verapamil and 6.7-fold in the single experiment with $[^{11}\text{C}]$GR205171.

$[^{11}\text{C}]$GR205171 was infused at a rate to achieve steady-state in both plasma and brain. The same study protocol as that in paper III was used, i.e. CsA was administered 20 min after the start of the $[^{11}\text{C}]$GR205171 infusion and the $[^{11}\text{C}]$GR205171 infusions were stopped at 70 min. $[^{11}\text{C}]$GR205171 plasma and brain concentrations reached equilibrium rapidly. Interestingly, the brain profile of $[^{11}\text{C}]$GR205171 looked very similar to that of $[^{11}\text{C}]$verapamil, Figure 11. It would be interesting to use a P-gp inhibitor other than CsA and repeat the experiments with $[^{11}\text{C}]$GR205171 and $[^{11}\text{C}]$verapamil to investigate whether the rapid increase in radiotracer concentration is caused by CsA or by the radiotracer itself. The BBB is highly permeable to both $[^{11}\text{C}]$GR205171 and $[^{11}\text{C}]$verapamil. The use of a P-gp substrate radiotracer with slower passive transport across the BBB could add information regarding the mechanism of P-gp inhibition. However, such a substrate might be less suitable as a PET radiotracer since it could require a longer study time. The PET method requires a substance to which the BBB
is highly permeable, although one to which the BBB was less permeable would be interesting from a scientific viewpoint. The development of new PET radiotracers for studying P-gp function will need to balance these two demands.

Species differences in blood-brain barrier transport

Species differences in the brain uptake of \([^{11}C]\)verapamil, \([^{11}C]\)GR205171 and \([^{18}F]\)altanserin in rat, guinea pig, minipig, monkey and human were investigated by comparing SUV, which is a concentration measurement adjusted for body weight and administered dose (Equation 13), and \(K_p\), which is the brain-to-plasma concentration ratio (Equation 14). A subgroup of the animals was administered with CsA to investigate possible species-related differences in P-gp function. ROIs were outlined in the cerebellum in all species since this region is devoid of 5HT\(_{2A}\) and NK\(_1\) receptors enabling investigation of BBB transport without interference of species differences in receptor binding. SUV and \(K_p\) values for all three studied radiotracers were clearly higher in humans, monkeys and minipigs than in rats and guinea pigs, as shown in Figure 12 and Table 5. SUV\(\text{increase}\) and \(K_p\text{increase}\) values with \([^{18}F]\)altanserin and \([^{11}C]\)GR205171 were higher in rats than in the other species. For \([^{11}C]\)verapamil, SUV\(\text{increase}\) values were similar in all species while \(K_p\text{increase}\) values were greater in rats than in guinea pigs or monkeys. However, since the plasma concentration is the driving force for brain concentrations, it was more relevant to compare \(K_p\text{increase}\) than SUV\(\text{increase}\) to obtain information on how much of the increase was due to P-gp inhibition rather than to changes in plasma pharmacokinetics.

We initially hypothesized that species differences in \(K_p\) and SUV were the result of differences in levels of expression of P-gp in the BBB or the capacity of P-gp to transport the substrates across the BBB. Differences in P-gp transport capacity could be explained by differing affinities of the substrate for P-gp. These differences in P-gp capacity or expression could, in fact, be an adequate explanation for the species differences observed in this study when no CsA was administered, i.e. when P-gp function was normal. If so, P-gp function would be more effective in guinea pigs than in rats, since \(K_p\) values were lower in guinea pigs than in rats, and more effective in rats than in monkeys, minipigs and humans. However, differences in P-gp capacity or expression do not explain the differences in \(K_p\text{increase}\). If it is assumed that the only difference between the two species is in P-gp capacity or expression, then the species with the lower baseline concentrations in the brain, in this case guinea pigs, should have a greater \(K_p\text{increase}\) value. However, \(K_p\text{increase}\) was lower in guinea pigs than in rats in this study, despite lower baseline concentration ratios in guinea pigs. Hence, our conclusion is
that the level of P-gp capacity or expression alone does not explain the observed species differences.

**Figure 12.** Sagittal PET images of $[^{11}C]$GR205171 in monkey, guinea pig and rat brain without (upper panel) and with (lower panel) administration of CsA 15 mg/kg + 5 mg/kg/h (monkey) or 22.5 mg/kg + 7.5 mg/kg/h (guinea pig and rat) starting 20 min prior to a bolus injection of $[^{11}C]$GR205171.

Cutler et al. demonstrated *in vivo* differences between rats and guinea pigs in the IC$_{90}$ of the P-gp inhibitor GF-120918 [117]. They showed that a higher plasma concentration of GF-120918 was needed in guinea pigs than in rats to achieve the same increase in brain concentrations of a P-gp substrate, SB-487946. *In vitro* studies have demonstrated differences in the capacity of P-gp-mediated transport in cell lines transfected with the MDR1 gene (gene coding for P-gp) from different species. In general, most of the evaluated substrates are transported to a higher degree by mouse or rat P-gp than by human P-gp [118-121]. Thus, both the transport capacity of P-gp and the IC$_{50}$ values of P-gp inhibitors may differ among species. This observation helps to explain our results. Species differences in the IC$_{50}$ of CsA, in combination with differences in the capacity or expression of P-gp, would explain the lower K$_{p, \text{increase}}$ values in guinea pigs than in rats, despite the lower baseline values in guinea pigs.

Plasma protein binding and metabolism affect the number of radiotracer molecules available for transport from the blood into the brain. However, both the degree of plasma protein binding and the metabolism of the studied
radiotracers were similar in all species and only small differences were observed between CsA-treated and untreated animals. Hence, neither metabolism nor plasma protein binding of the radiotracers seem to be adequate explanations for the observed species differences.

Table 5. Average SUV, $K_p$, SUV$_{\text{increase}}$ and $K_p,\text{increase}$ values over 60 min in the cerebellum for the studied radiotracers. Standard deviation is given in parenthesis if $n \geq 3$, ns=not significant, $^*p < 0.05$, $**p < 0.01$, and $***p < 0.001$

<table>
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<th>Radiotracer</th>
<th>Species</th>
<th>SUV$_{\text{baseline}}$</th>
<th>SUV$_{\text{CsA}}$</th>
<th>SUV$_{\text{increase}}$</th>
<th>$K_p,\text{baseline}$</th>
<th>$K_p,\text{CsA}$</th>
<th>$K_p,\text{increase}$</th>
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</thead>
<tbody>
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<td>[11C]verapamil</td>
<td>Rat</td>
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<td>0.85 (0.50)</td>
<td>4.1*</td>
<td>1.13 (0.36)</td>
<td>6.52 (1.71)</td>
<td>5.8**</td>
</tr>
<tr>
<td></td>
<td>Guinea pig</td>
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<td>0.54 (0.08)</td>
<td>3.9***</td>
<td>0.65 (0.02)</td>
<td>1.89 (0.57)</td>
<td>2.9**</td>
</tr>
<tr>
<td></td>
<td>Monkey</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[11C]GR205171</td>
<td>Rat</td>
<td>0.32 (0.05)</td>
<td>1.16 (0.24)</td>
<td>3.6**</td>
<td>3.45 (0.52)</td>
<td>11.9 (3.7)</td>
<td>3.5**</td>
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<tr>
<td></td>
<td>Guinea pig</td>
<td>0.20 (0.05)</td>
<td>0.45 (0.06)</td>
<td>2.3**</td>
<td>2.13 (0.83)</td>
<td>4.31 (2.89)</td>
<td>2.0**</td>
</tr>
<tr>
<td></td>
<td>Monkey</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>Human</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>[18F]altanserin</td>
<td>Rat</td>
<td>0.32 (0.11)</td>
<td>0.83 (0.46)</td>
<td>2.6*</td>
<td>0.23 (0.10)</td>
<td>0.54 (0.33)</td>
<td>2.3*</td>
</tr>
<tr>
<td></td>
<td>Minipig</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Human</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

$^a$Ex-vivo studies; values measured at 60 min after radiotracer administration.

Differences in the intra-brain distribution, $V_{u,br}$, between species could also be relevant to our results. The total substrate brain concentrations observed with PET will depend on the binding potential of the substrate to brain tissue. In this instance, the term binding is taken to include both specific binding to receptors and nonspecific binding to brain tissue components. Brain concentrations of the radiotracer will be higher in species with a higher binding potential to brain tissue than in those with a lower binding potential. Thus, species differences in intra-brain distribution could explain the SUV and $K_p$ differences observed in our study when P-gp function was uninhibited while species differences in the IC$_{50}$ of CsA could explain the differences in the magnitude of the increase in brain concentrations after
CsA treatment. Species differences in passive transport over the BBB would lead to the same outcome as differences in the intrabrain distribution since both active and passive transport contribute to the $K_p$. However, it is unlikely that the physicochemical properties of, and tightness of tight junctions in, the BBB itself would differ to a large extent among species [122]. Further, the expression of other active efflux transporters such as Mrp, Oat, and Oatp could contribute to the species differences noted.

Liow et al. speculated that P-gp inhibition caused a smaller increase in [11C]RWAY concentrations in the cerebellum than in 5HT$_{1A}$ receptor-rich regions after CsA administration [123]. Our results showed a similar trend in regional differences; SUV$_{\text{increase}}$ and $K_p,\text{increase}$ values were somewhat smaller in the cerebellum than in the high binding regions of the striatum and frontal cortex for [11C]GR205171 and [18F]altanserin, respectively (paper V).

Single bolus and bolus plus infusion regimens in PET studies

The studies in paper II-V have utilized a single bolus dose or a bolus dose followed by infusions, Table 2. There are several advantages and disadvantages with both methods. For a reversible radiotracer, the concentrations in brain and plasma usually become parallel over time during the elimination phase after a single bolus. However, as Carson et al. have demonstrated, this apparent $K_p$ is different from the $K_p$ measured at true equilibrium [124]. For example, if the elimination rate from plasma is faster than the elimination rate from brain, the apparent $K_p$ will be larger than the true $K_p$. $K_p$ can be directly calculated in an infusion study from the steady-state concentrations in brain and plasma. $K_p$ can, however, also be estimated from bolus experiments if pharmacokinetic modelling is applied, by calculating the ratio of the rate constants, $k_{in}/k_{out}$ for a one-compartment brain model or $k_{in}/k_{out}(1 + k_3/k_4)$ for a two-compartment model, or from the slope of a Logan graphical analysis [125]. Hence, $K_p$ determination based on infusion studies is model-independent, while accurate $K_p$ determination in bolus studies requires pharmacokinetic modelling. Since all models have limitations and include a number of assumptions and simplifications (“all models are wrong but some are still useful” as stated by George Box in 1973) it is a clear advantage to have an experimental paradigm which allows calculation of $K_p$ with minimal complexity in the model. Further, in PET, tracer synthesis is often resource-demanding. A bolus plus infusion protocol requires only one delivery for determining the effect of a pharmacological intervention, as described with CsA in this thesis and for several other PET intervention studies at receptor level [124,126-131]. The effect of the intervention drug can
be seen explicitly in one single subject. The subject can then be used as its own control; however, in a bolus study, one experiment is a control/baseline experiment and the other is the intervention experiment. Sometimes it is not possible to use the same individual in the bolus control as in the bolus intervention experiments. Hence, it is difficult to know whether the difference between the control and the intervention is due to intersubject variability or to an effect of the intervention drug. On the other hand, an infusion regimen requires a number of bolus experiments for determination of the optimal infusion scheme. Ideally, a subject-specific infusion should be designed, based on the individual pharmacokinetics, but this is not usually possible. Instead, infusion rates based on typical pharmacokinetic parameter values are normally used. One drawback is, of course, that variations between individuals could result in non-steady-state concentrations and hence confound the results. Most infusion studies are performed as a single bolus followed by a constant infusion. For example, CsA was administered in this manner in paper III and V. However, we chose to use exponential stepwise infusions of the radiotracers in paper II, III and IV. The advantage is that steady-state is reached faster than with a bolus followed by a constant infusion.

Another advantage of using an infusion protocol is the reduction of the fraction of radiolabelled metabolites, as intact radiotracer is infused throughout the study.

Infusion experiments are, however, technically more difficult than single bolus experiments. An infusion experiment requires cannulation of the vein during the whole experiment while a bolus only requires one single injection into the blood. Further, the optimal time intervals for baseline and, if an intervention drug is used, intervention states must be determined prior to the infusion study. This generally results in a trade-off between achieving equilibrium and maximizing statistical counts.

Time aspect is important in the design of transporter inhibition studies. In the discussion around P-gp (and other transporters) the main issue has often been whether or not a drug is a substrate, and whether or not it is an inhibitor. The dynamics of the inhibition process have thus not been addressed in most studies. PET studies on P-gp modulation have often resembled receptor studies in this respect [132,20,123]. Hence, the blocking agent (e.g. CsA) has been given 30 min or more before a bolus of the radiotracer (e.g. [11C]verapamil). Drug interaction with the active transporters at the BBB might lead to a rapid concentration increase in the brain when the transporters become blocked, even if the efflux function is restored rapidly again. This behaviour will not be picked up if the inhibitor is given before the marker. By giving the marker as an infusion to achieve steady-state before the inhibitor was given, it was possible to follow both the onset and the decline of P-gp inhibition.

The reason for not using an infusion protocol for the radiotracers in paper V was that some of the clinical data already existed and more accurate com-
Comparisons could be done between species if all studies were performed according to the same protocol. It would also have been rather resource demanding to first perform bolus experiments for each species and radiotracer to calculate the infusion rates before actually performing the intervention experiments.

Comparison of MATLAB and NONMEM

PET pharmacokinetic modelling is usually performed using MATLAB programs that are developed in house. The advantage is that they can be written to suit the specific purpose of a study, but the disadvantage is the low level of validation. The software PMOD has also been used for PET pharmacokinetic modelling [133]. In classical pharmacokinetic/pharmacodynamic modelling, several different types of software are used, but these are still rarely utilized with PET data. For example, the population pharmacokinetic modelling software NONMEM has only been used for modelling a handful of PET studies [134-137]. NONMEM resolves both intra- and inter-subject variability, i.e. it gives a description of the pharmacokinetics in the typical subject as well as information about variation in the study population.

NONMEM was used for pharmacokinetic modelling in paper III, while MATLAB was used in paper II. Both approaches lead to similar results, i.e. the same type of model describing the transport of $[^{11}\text{C}]$verapamil across the BBB was found to be optimal (CsA affected transport of $[^{11}\text{C}]$verapamil out of the brain). The parameter values for both the BBB model and the indirect effect model obtained with MATLAB and NONMEM were similar. When NONMEM was used, inter-animal variation was only found for the IC$_{50}$ parameter. The other model parameters were described by the typical parameter value, i.e. one parameter value could be used for all individuals, just as with the parameter values obtained with MATLAB. Thus, MATLAB and NONMEM performed equally well when modelling the pharmacokinetics of the $[^{11}\text{C}]$verapamil-CsA interaction at the BBB. The rats receiving the same dose of CsA displayed very similar brain concentrations of $[^{11}\text{C}]$verapamil. Animals, especially rodents, used in preclinical studies are bred to be genetically very similar to each other, so the low inter-animal variation is perhaps not so surprising. The number of experiments in PET is often small and this may be a reason why no inter-animal variation was found for most of the pharmacokinetic parameters. Mixed effects modelling may contribute more to the understanding of the pharmacokinetics in clinical PET studies where the differences between subjects and inter-occasion differences are more pronounced.
Conclusions

A central aspect of this thesis has been to explore the novel combination of dynamic PET imaging of labelled drug entering the brain, administered by an exponential stepwise infusion scheme which allows time-resolved consequence analysis of P-gp inhibition, and mathematical modelling of the obtained dynamic data.

The inhibition of P-gp by CsA was very rapid and was also rapidly reversible when CsA was eliminated; the restoration of P-gp function was detected quickly due to the fast brain pharmacokinetics of $[^{11}\text{C}]$verapamil. Therefore, it can be concluded that $[^{11}\text{C}]$verapamil is a good substrate for P-gp interaction studies in the brain using PET, as the concentrations in brain and plasma rapidly equilibrate, enabling fast detection of changes in P-gp function. Another advantage is that the brain-to-plasma ratio of $[^{11}\text{C}]$metabolites is unaffected by P-gp inhibition.

The P-gp inhibition was best described by an inhibitory indirect effect model in which CsA decreased the transport of $[^{11}\text{C}]$verapamil out of the brain. The studies showed that around 90% of the transport of $[^{11}\text{C}]$verapamil out of the brain was P-gp-mediated. This investigation thus indicated that P-gp is acting on efflux rather than influx mechanisms. Further, as P-gp is situated at the luminal membrane, it is likely that the substrate molecules are taken up in the endothelial cells, i.e. before they have entered the brain, and then transported back to the plasma. The studies presented in this thesis demonstrated the importance of including the dynamics of the inhibition in the experimental design and that studies of efflux transporters should preferably be performed using steady-state concentrations of the marker followed by an intervention with the inhibitor. This method is applicable to drugs under development and can be used not only in rodents but also in higher species, potentially even in humans, to understand the effect of P-gp or other transporters on the uptake of drugs in the brain.

The simulation of active efflux at the BBB showed that influx hindrance is more effective than efflux enhancement in decreasing brain concentrations of a substrate. If transport across the BBB is slower than elimination from plasma, a change in the elimination half-life in the brain after transporter inhibition indicates active transport by the efflux enhancement mechanism. On the other hand, if the half-life is unchanged after inhibition, this may indicate influx hindrance. Further, as the bulk flow is small, most compounds will display a $K_{p,uu}$ of 1 if they are unaffected by active efflux.
The unbound volume of distribution or the permeability per se will not affect the extent of brain uptake, i.e. the \( K_{puu} \), but will affect the time it takes to reach equilibrium between unbound concentrations in plasma and brain. Hence, a hydrophilic molecule may have good central effects if it is not effluxed actively; it is just a question of the time required to reach therapeutic concentrations in the brain.

The hydroxyurea study further supported these findings, and helped to explain why the extent and rate of brain uptake are two separate phenomena. Hydroxyurea was not found to be a substrate for P-gp or a probenecid-sensitive transporter, although the brain concentrations during the study period were low. The low brain concentrations were rather the result of slow transport across the BBB. However, hydroxyurea is an example of a drug that is administered repeatedly and will therefore have time to penetrate the various biological membranes, including the BBB, to produce its therapeutic effect. Nonetheless, the compound is not suitable as a PET radiotracer since it did not reach equilibrium during the investigation time.

The study on species differences in transport across the BBB showed that brain drug concentrations may differ several-fold between species. The studied radiotracers showed lower concentrations in rodents than in humans, monkeys and pigs. The increase in brain concentrations after P-gp inhibition was somewhat greater in rats than in the other species. The combined effects of the expression or capacity of P-gp and the inhibition potency of CsA are likely to play an important role, although BBB permeability and differences in intrabrain substrate distribution may also contribute to species differences. The investigation showed that compounds that are P-gp substrates in rodents are likely to be substrates in higher species as well.

*In vitro* methods for screening P-gp-mediated transport and *in vivo* animal models are usually utilized in drug development before the drug candidates are studied in *in vivo* experiments in humans. If a drug intended for a target inside the brain is found to be a P-gp substrate in *in vitro* experiments or to have low brain concentrations in a rat model due to it being a P-gp substrate, it might simply be discarded from further development. This is unfortunate, as such compounds could still reach clinically sufficient brain concentrations in humans. Nonetheless, drugs that are P-gp substrates should be studied carefully with respect to drug-drug interaction at the BBB since their brain concentrations might increase if they are used together with a P-gp inhibitor drug. However, our results indicate that the dosages of CsA used clinically, e.g. before and after organ transplantation, would only result in a doubling of the verapamil brain concentrations. Hence, it remains to be seen whether P-gp inhibition at the BBB is clinically significant.
Populärvetenskaplig sammanfattning på svenska


För att bota ovannämnda sjukdomar eller lindra de symptom de orsakar behöver vi läkemedel som verkar på den plats i kroppen som är drabbad, dvs hjärnan. Blod-hjärnbarriärens skydd, till exempel transportörerna, kan ofta inte se skillnad på skadliga främmande ämnen och läkemedel. Därför hindrar dessa transportörer även läkemedel från att nå fram till sina målsystem (receptorer, enzymer, tumörceller etc) i hjärnan.

Det övergripande målet med detta avhandlingsarbete var att studera transport över blod-hjärnbarriären med hjälp av positronemissionstomografi (PET) samt att med matematiska simuleringar och modellering försöka förstå och beskriva transporten. I samband med detta utvecklades även nya PET-metoder för transportörstudier.

Studierna som presenteras i delarbete två, tre och fyra utfördes på råtor. De flesta transportörer, inklusive P-glykoprotein, återfinns även hos människa. I det femte delarbetet jämfördes koncentrationer av tre PET-spårsubstanser, som alla visat sig vara P-glykoproteinsubstrat, i rätta, marsvin, gris, apa och människa.


Jämförelsen mellan koncentrationer av tre olika PET-spårsubstanser i olika arter visade att gnagare, dvs rätta och marsvin, hade upp till 10 gånger lägre koncentrationer i hjärnan än högre arter, dvs gris, apa och människa. Ökningen i koncentration då P-glykoprotein inhiberades med hjälp av cyklosporin A var lika stor i alla arter. En läkemedelskandidat som visat sig vara ett P-glykoproteinsubstrat i rätta är därför antagligen även det i människa. Det är dock fortfarande oklart vilken klinisk betydelse det är om ett läkemedel är ett transportörsubstrat. Den matematiska modellen för interaktionen mellan cyklosporin A och $[^{11}\text{C}]$verapamil visade att den största möjliga ökningen av $[^{11}\text{C}]$verapamil-koncentrationen i hjärnan, det vill säga då P-glykoprotein är helt inhiberat, är ca 15 gånger. Denna maximala ökning uppnås vid cyklosporin A koncentrationer som är mycket högre än de koncentrationer som erhålles efter doser som administreras i kliniskt bruk vid till exempel organtransplantationer. Modellen indikerade att kliniska koncentrationer av cyklosporin A endast kan leda till en dubblering av $[^{11}\text{C}]$verapamil koncentrationen i hjärnan.
Artskillnader är viktiga att beakta vid utveckling av nya läkemedel. Risken finns att potentiellt fungerande läkemedel förkastas efter försök i små försöksdjur om man utgår från att koncentrationer direkt kan extrapoleras till människa.
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A doctoral dissertation from the Faculty of Pharmacy, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy”.)