Role of the Blood-Brain Barrier in Stereoselective Distribution and Delay in $H_1$ Receptor Occupancy of Cetirizine in the Guinea Pig Brain

ANUBHA GUPTA
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Abstract

Cetirizine, an H1-antihistamine, is prescribed for allergic disorders. It exists as a racemic mixture, with levocetirizine being the active enantiomer. The central nervous system side-effects of H1-antihistamines are caused by their penetration into the brain. In this thesis the plasma pharmacokinetics, transport across the blood-brain barrier (BBB) and H1 receptor occupancy of cetirizine enantiomers was investigated in vivo in guinea pigs. The transport across the BBB was quantified using the microdialysis technique. Stereoselective brain distribution was investigated by measuring both unbound and total concentrations in plasma and brain. The time aspects of the H1 receptor occupancy of levocetirizine was studied in the brain and the periphery.

The plasma pharmacokinetics of cetirizine was stereoselective with clearance and volume of distribution of levocetirizine being approximately half that of dextrocetirizine. This was mainly due to the differences in plasma protein binding of the enantiomers. The stereoselectivity in brain distribution indicated by the partition coefficient Kp (total AUC ratio brain to plasma) was caused by stereoselective plasma protein binding. The transport across the BBB measured in this thesis by the unbound partition coefficient Kp,unb (unbound AUC ratio brain to plasma) was the same for the two enantiomers. Binding within the brain was also not significantly different. The H1 receptor occupancy of levocetirizine in brain lagged behind the plasma concentrations whereas it was not delayed with respect to the brain concentrations. This indicates that the delayed brain H1 receptor occupancy of levocetirizine is caused by a slow transport across the BBB.

In summary, the results of this thesis emphasize the importance of measuring both the unbound and total concentrations in blood and brain to characterize stereoselective brain distribution. The thesis also emphasize the importance of taking local brain pharmacokinetics into consideration in understanding pharmacokinetic-pharmacodynamic relationships of drugs with central activity.

Keywords: Cetirizine enantiomers, Brain distribution, Microdialysis, H1 receptor occupancy, Stereoselective-pharmacokinetics

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urn:nbn:se:uu:diva-6360 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-6360)
To my family
Faculty opponent: Dr Joseph W. Polli
Drug Metabolism and Pharmacokinetics, GlaxoSmithKline, Inc,
Research Triangle Park, NC, USA.
Papers Discussed

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


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Abbreviations

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<tr>
<td>Abcb1b−/−</td>
<td>P-glycoprotein knockout mice</td>
</tr>
<tr>
<td>Abcb1b+/+</td>
<td>Wild type mice</td>
</tr>
<tr>
<td>$A_{br}$</td>
<td>Total amount of drug in the brain</td>
</tr>
<tr>
<td>AGP</td>
<td>Alpha acid glycoprotein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the concentration-time curve</td>
</tr>
<tr>
<td>AUC$_{u}$</td>
<td>Area under the unbound concentration-time curve</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>$C_{bi}$</td>
<td>Concentration in the blood</td>
</tr>
<tr>
<td>$C_{ia}$</td>
<td>Concentration in the microdialysis perfusate</td>
</tr>
<tr>
<td>CL</td>
<td>Clearance</td>
</tr>
<tr>
<td>$CL_{in}$</td>
<td>Influx clearance from blood to brain</td>
</tr>
<tr>
<td>$CL_{u}$</td>
<td>Unbound clearance</td>
</tr>
<tr>
<td>$C_{ex}$</td>
<td>Concentration in the external medium ((in \text{ vitro}))</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>$C_{iex}$</td>
<td>Concentration in the microdialysis dialysate</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>$C_{u,brISF}$</td>
<td>Unbound concentration in brain interstitial fluid</td>
</tr>
<tr>
<td>CUP</td>
<td>Cuprophane</td>
</tr>
<tr>
<td>CZE</td>
<td>Cetirizine</td>
</tr>
<tr>
<td>$E_{max}$</td>
<td>Maximum effect</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>FEP</td>
<td>Fluorinated ethylene propylene</td>
</tr>
<tr>
<td>FOCE</td>
<td>First order conditional estimate</td>
</tr>
<tr>
<td>$f_{u,brIF}$</td>
<td>Ratio of unbound concentrations in brain interstitial fluid to the total amount per gram brain tissue</td>
</tr>
<tr>
<td>$f_{u,pl}$</td>
<td>Fraction unbound in plasma</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>IIV</td>
<td>Interindividual variability</td>
</tr>
<tr>
<td>IS</td>
<td>Internal standard</td>
</tr>
<tr>
<td>ISF</td>
<td>Interstitial fluid</td>
</tr>
<tr>
<td>$k_{e}$</td>
<td>Elimination rate constant from systemic circulation</td>
</tr>
<tr>
<td>$K_{in}$</td>
<td>Transfer coefficient ((volume/time/weight \text{ brain}))</td>
</tr>
<tr>
<td>$k_{e,brain}$</td>
<td>Elimination rate constant from brain</td>
</tr>
<tr>
<td>$K_p$</td>
<td>Equilibrium distribution ratio of drug between tissue and blood or plasma based on total concentrations in blood and tissue</td>
</tr>
<tr>
<td>$K_{p,brain}$</td>
<td>Equilibrium distribution ratio of drug between tissue and blood or plasma based on unbound concentration in blood and total concentration in tissue</td>
</tr>
<tr>
<td>$K_{p,brain}$</td>
<td>Equilibrium distribution ratio of drug between tissue and blood or plasma based on unbound concentrations in blood and tissue</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>LC/MS/MS</td>
<td>Liquid chromatography tandem mass spectrometry</td>
</tr>
<tr>
<td>LLOQ</td>
<td>Lower limit of quantification</td>
</tr>
<tr>
<td>M6G</td>
<td>Morphine-6-glucuronide</td>
</tr>
<tr>
<td>MF1</td>
<td>Individual multiplication factor 1</td>
</tr>
<tr>
<td>MF2</td>
<td>Multiplication factor 2</td>
</tr>
<tr>
<td>n.s.</td>
<td>Not significant</td>
</tr>
<tr>
<td>NONMEM</td>
<td>Nonlinear mixed effects modeling</td>
</tr>
<tr>
<td>NSB</td>
<td>Nonspecific radioligand binding</td>
</tr>
<tr>
<td>OAT</td>
<td>Organic anion transporter</td>
</tr>
<tr>
<td>OFV</td>
<td>Objective function value</td>
</tr>
<tr>
<td>PC</td>
<td>Polycarbonate</td>
</tr>
<tr>
<td>pD2</td>
<td>The negative logarithm to base 10 of an agonist concentration producing 50 % of maximum possible effect</td>
</tr>
<tr>
<td>PAES</td>
<td>Polyarylethersulphone</td>
</tr>
<tr>
<td>PBPK</td>
<td>Physiology based pharmacokinetic model</td>
</tr>
<tr>
<td>PES</td>
<td>Polyethersulphone</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PS</td>
<td>Permeability surface area product</td>
</tr>
<tr>
<td>QCH</td>
<td>Quality control high</td>
</tr>
<tr>
<td>QCL</td>
<td>Quality control low</td>
</tr>
<tr>
<td>QCM</td>
<td>Quality control medium</td>
</tr>
<tr>
<td>R-CZE</td>
<td>Levocetirizine</td>
</tr>
<tr>
<td>RSB</td>
<td>Radioligand specific binding</td>
</tr>
<tr>
<td>RSE</td>
<td>Relative standard error</td>
</tr>
<tr>
<td>S-CZE</td>
<td>Dextrocetirizine</td>
</tr>
<tr>
<td>SLC</td>
<td>Solute carrier transporters family</td>
</tr>
<tr>
<td>( t_{1/2} )</td>
<td>Terminal half-life</td>
</tr>
<tr>
<td>( V )</td>
<td>Volume of distribution</td>
</tr>
<tr>
<td>( V_{b} )</td>
<td>Volume of blood in the brain</td>
</tr>
<tr>
<td>( V_{ss} )</td>
<td>Volume of distribution at steady state</td>
</tr>
<tr>
<td>( V' )</td>
<td>Unbound volume of distribution</td>
</tr>
<tr>
<td>( V_{ub} )</td>
<td>Unbound volume of distribution in the brain</td>
</tr>
<tr>
<td>( \lambda )</td>
<td>Terminal rate constant</td>
</tr>
</tbody>
</table>
Introduction

Distribution of drugs to the brain is restricted by the presence of the blood-brain barrier (BBB). The BBB is not only a physical barrier but also express many transporters involved in the influx and efflux of drugs (Kusuhara and Sugiyama, 2005; Loscher and Potschka, 2005; Tsuji, 2005). Understanding of the role of the BBB in the pharmacokinetics and pharmacodynamics is important during development of drugs with central nervous system (CNS) effects or side-effects. The necessity of more research in this area has recently been reviewed (Pardridge, 2005).

H₁-antihistamines are a category of drugs for which the entry into the brain should be avoided to minimize side-effects. Some H₁-antihistamines have higher incidence of CNS side-effects compared to others and a lot of research has focused to understand these differences. The difference lies in the extent to which these drugs enters into the CNS. Physicochemical properties of drugs are important, but not the only factor affecting the brain penetration (Timmerman, 1999). It is only recently that the importance of BBB efflux transporters in the low penetration of these drugs has been realized (Cvetkovic et al., 1999; Tamai et al., 2000; Chishty et al., 2001; Chen et al., 2003; Polli et al., 2003; Ishiguro et al., 2004). In this thesis, cetirizine (CZE), a second-generation H₁-antihistamine, was used as a model drug for the H₁-antihistamine group to understand the role of the BBB in its pharmacokinetics and pharmacodynamics in the brain.

One of the important aspects of brain distribution is the extent of transport across the BBB. Parameters commonly used in the literature, $K_p$ and $K_{pu}$ were compared with the parameter termed as $K_{pu,u}$ in this thesis. Stereoselective plasma pharmacokinetics and brain distribution of CZE were also investigated with emphasis on the understanding of the factors that could lead to stereoselective brain distribution. The role of BBB transport for the delay in levocetirizine pharmacodynamics in the brain was investigated. The histamine H₁ receptor occupancy measured ex-vivo was used as a surrogate biomarker of levocetirizine pharmacodynamics in the brain (Yanai et al., 1999).

H₁-antihistamines and Their CNS Side-effects

H₁-antihistamines are drugs of choice for treatment of various allergic disorders. The antiallergic effect is mainly due to antagonism of histamine in the periphery, which is an important mediator of allergic reactions (Simons and Simons, 1991).
In the CNS, histamine is associated with a wide range of functions such as arousal, cognition, regulation of sleep wake cycle, learning and memory, mediated mainly through histamine H₁ receptors (Watanabe and Yanai, 2001). The antagonism of H₁ receptors in the brain, therefore, leads to side-effects like sedation and cognitive impairment. Based on the side-effects caused by antihistamines they are classified into first- and second-generation antihistamines. Second-generation antihistamines have lower incidence of these side-effects because of their limited brain distribution. CZE is a second-generation H₁-antihistamine and exists as a racemic mixture. The pharmacological activity of CZE is mainly attributed to levocetirizine (R-CZE) (Devalia et al., 2001). In binding assays, R-CZE has demonstrated approximately 30-fold higher affinity than dextrocetirizine (S-CZE). The difference in affinities between the two enantiomers is mainly accounted for by their different dissociation rates from H₁ receptors, with R-CZE demonstrating a far longer dissociation half-life (142 min) than S-CZE (6 min) (Gillard et al., 2002). Any difference in the BBB transport of CZE enantiomers is not known.

Brain Physiology and the Blood-brain Barrier

Three barrier layers limit and regulate molecular exchange at the interfaces between the blood and the neural tissue: the BBB, formed by cerebrovascular endothelial cells between the blood and the brain interstitial fluid (ISF), the choroid plexus epithelium between the blood and the ventricular cerebrospinal fluid (CSF) and the arachnoid epithelium between the blood and the subarachnoid CSF (Figure 1).

Figure 1. Locations of the barriers in the CNS. (1) the brain endothelium forming the blood–brain barrier (BBB), (2) the arachnoid epithelium forming the middle layer of the meninges, and (3) the choroid plexus epithelium which secretes cerebrospinal fluid (CSF). In circumventricular organs (CVO), containing neurons specialised for neurosecretion and/or chemosensitivity, the endothelium is leaky. This allows tissue-blood exchange, but as these sites are separated from the rest of the brain by an external glial barrier, and from CSF by a barrier at the ependyma, CVOs do not form a leak across the BBB (based on Segal and Zlokovic, 1990 modified by Dr A Reichel for Abbott, 2004).
These barriers are crucial for the maintenance of cerebral homeostasis. The BBB is characterized by the presence of tight junctions between the endothelial cells, absence of fenestrations and few pinocytotic vesicles. After systemic drug administration, the BBB is likely to be more important for drug delivery to the brain due to larger surface area and the closeness between brain capillaries. In order to reach brain ISF, the molecules have to cross two membranes of the BBB endothelial cells, one facing blood (luminal) and the other facing brain ISF (abluminal). Various transport processes involved in the drug transport across the BBB are described below (Figure 2).

**Transport Processes Across the Blood-brain Barrier**

**Passive Diffusion**

Passive diffusion is an energy independent transport of unbound molecules across a cell membrane. This process involves the movement of molecules along the concentration gradient. It can occur via the paracellular (between the cells) and/or the transcellular route (across the cells). Due to the presence of tight junctions transcellular passive diffusion is the most important transport process for small lipophilic molecules. All other molecules either are very slowly transported across the BBB, or are transported via catalyzed transport, owing to the specific interaction between the drug and certain BBB transport systems.

**Carrier Mediated Transport**

Carrier mediated transport involves a membrane protein (transporter) in the transport process. The substrate binds to a transporter and the resulting complex undergoes a change in conformation that permits the substrate to traverse the membrane and be released on the other side. Carrier mediated transport is characterized by saturability and specificity. For example an amine transport system was demonstrated for H₁-antihistamines (Yamazaki et al., 1994; Yamazaki et al., 1994). They showed...
Anubha Gupta

Saturable uptake of the classical H₁-antihistamine [³H]mepyramine into the brain using monolayers of primary cultured bovine brain capillary endothelial cells. The uptake was inhibited by amine drugs such as chlorpheniramine but not by choline or anionic drugs. Several, H₁-antagonists, azelastine, ketotifen, cyproheptadine, emedastine, and CZE, are also likely taken up by a common mechanism, based on observed mutual inhibitory effects. However, CZE had least inhibitory effect among them (Yamazaki et al., 1994). The carrier mediated transport can be facilitated diffusion or active transport as described below.

**Facilitated Diffusion**
Facilitated diffusion involves transporters which allow passage of substrates across membranes down their concentration gradients e.g. GLUT1 and System L for transport of D-glucose and large neutral amino acids (phenylalanine), respectively (Tsuji and Tamai, 1999). Involvement of GLUT1 in the transport of M6G (Bourasset et al., 2003) and System L in the transport of gabapentin has been proposed (Welty et al., 1993).

**Active Transport**
Active transport involves transporters that utilize energy to transport substrates against a concentration gradient. Depending on the source of energy the active transport could be primary or secondary-active transport (Hediger et al., 2004). Primary-active transport process uses ATP as source of energy. For example P-glycoprotein (P-gp) belongs to a class of ATP-binding cassette transporters family. It is located on the luminal membrane of the BBB and is involved in efflux of various drugs (Tsuji et al., 1992; Schinkel, 1999).

Secondary-active transport process uses energy from an ion gradient. OAT3 is a secondary active transporter and belongs to SLC (Solute carrier) family. It is located at the abluminal membrane and is involved in the efflux of p-aminohippuric acid and benzylpenicillin across the BBB (Kikuchi et al., 2003). Based on the direction of transport across the BBB, transporters are termed as efflux (brain to blood) or influx (blood to brain) transporters.

The transport processes involved in the delivery of a drug to the brain influence its brain pharmacokinetics (Hammarlund-Udenaes et al., 1997). Various components of the pharmacokinetics of brain delivery are described below.

**Pharmacokinetic Aspects of Brain Drug Delivery**
The pharmacokinetics of brain drug delivery has three components: rate of transport, extent of transport and binding within the brain.
Rate of Drug Delivery to the Brain

The rate of transport across the BBB is generally estimated as the permeability across the BBB also called the PS product, or the influx clearance ($K_{in, CL_{in}}$). It is generally measured in μl/min/g brain. This is a clearance measurement and not a rate per se. The measurement describes the net capacity of the BBB to transport a drug into the brain. The presence of efflux transporters like P-gp on the luminal side decreases the permeability (net influx clearance) in relation to the passive permeability (Dagenais et al., 2004), while active influx would increase the permeability in relation to passive permeability. Another parameter measuring the rate of brain equilibration, has recently been described by Liu et al. It was defined as the half-life ($t_{1/2eq,in}$) for free brain concentration to equilibrate with free plasma concentration. Using a PBPK model, it was shown to be dependent on PS product and the nonspecific binding in the brain (Liu et al., 2005).

Extent of Drug Delivery to the Brain

The amount of drug present in the brain at steady state in relation to that in blood is the other important aspect of drug delivery to the brain. Brain concentrations are generally compared with blood or plasma concentrations to estimate the delivery of drug to the brain. This ratio, $K_p$, can be estimated either as ratio of steady state concentrations or AUC.

\[
K_p = \frac{AUC_{tot,br,0-\infty}}{AUC_{tot,pl,0-\infty}}
\]

where $AUC_{tot,br,0-\infty}$ and $AUC_{tot,pl,0-\infty}$ are the areas under the curve of total concentrations vs. time in brain and plasma, respectively. The $K_p$ can also be expressed as

\[
K_p = \frac{f_{u,pl} * AUC_{u,brISF,0-\infty}}{f_{u,brISF} * AUC_{u,pl,0-\infty}}
\]

where $AUC_{u,brISF,0-\infty}$ and $AUC_{u,pl,0-\infty}$ are the areas under the curve of unbound concentrations vs. time in brain ISF and plasma, respectively. The $f_{u,pl}$ is the unbound fraction of the drug in plasma and $f_{u,brISF}$ is the ratio of unbound concentrations in brain ISF to the total amount per gram brain tissue. The unbound brain ISF concentrations can be obtained using the microdialysis technique described later. Thus, the $K_p$ value is a composite of the BBB equilibrium and the tissue and protein bindings in brain and blood.

To compensate for differences in plasma protein binding the partition coefficient $K_{p,u}$ can be calculated as:
or expressed differently

\[ K_{p,u} = \frac{AUC_{u,\text{brISF},0\rightarrow\infty}}{\int_{u,\text{brISF}} AUC_{u,pl,0\rightarrow\infty}} \]  

Thus \( K_{p,u} \) is a composite of the BBB equilibration and binding within the brain.

The ratio of the area under the unbound brain ISF concentration time profile and area under the unbound plasma concentration time profile was calculated to compensate for the binding also within the brain tissue. This ratio in analogy to the partition coefficients above is called the unbound partition coefficient \( K_{p,uu} \) and directly describe the equilibrium across the BBB:

\[ K_{p,uu} = \frac{AUC_{u,\text{brISF},0\rightarrow\infty}}{AUC_{u,pl,0\rightarrow\infty}} \]  

The unbound partition coefficient \( K_{p,uu} \) between any tissue and blood can give valuable quantitative information on the \textit{in vivo} capacity of active efflux or influx at the membrane separating the tissue from blood. The information obtained is an \textit{in vivo} measurement which gives the net effect of all transporters that might be involved. If the BBB transport of a molecule is dominated by passive transport, \( K_{p,uu} \) will be 1, e.g. codeine (Xie and Hammarlund-Udenaes, 1998). \( K_{p,uu} > 1 \) indicates that active influx dominates at the BBB as e.g. for apomorphine (Sam et al., 1997) and gacyclidine (Hoizey et al., 2000). A value < 1 indicates active efflux at the BBB e.g. morphine (Tunblad et al., 2003). However, processes other than efflux at the BBB can also lead to \( K_{p,uu} < 1 \). The ISF in the brain flows along perivascular spaces and axon tracts, draining into the CSF compartment (Abbott, 2004). This flow termed as bulk flow likely contributes to elimination of low permeability drugs from the brain. Cerebral metabolism can also lead to lower unbound concentrations in the brain (Ravindranath and Boyd, 1995). However, there is currently no quantitative data on the extent to which drugs are metabolized within the CNS. In general, the metabolic capacity in the liver likely contributes more to metabolite presence in the brain by direct transport across the BBB.

**Binding Within the Brain Tissue**

Once the drug has reached brain ISF it can exist as unbound in ISF or bound to both extracellular and intracellular components. An estimate of binding within the brain can be made using the parameter \( V_{u,br} \), the unbound volume of distribution in brain expressed as
where $A_{br}$ is the total amount of the drug per gram brain, $V_{bl}$ is the volume of blood per gram brain, $C_{bl}$ is the total concentration in blood, and $C_{u,brISF}$ is the unbound concentration in brain ISF (Wang and Welty, 1996). As $V_{u,br}$ has the free drug in brain ISF as the reference ($C_{u,brISF}$), the obtained values can be compared with physiological volumes. The brain extracellular space is reported as 0.12-0.20 ml/g brain (Levin et al., 1970; Goodman et al., 1973). Thus, a volume close to 0.2 ml/g brain shows that the drug is mainly distributed into brain ISF and a higher value indicates that drug is either distributed intracellularly and/or binds to protein in ISF. It is to be noted that this parameter is different from the volume term used in PET or in situ brain perfusion studies.

Another pharmacokinetic parameter which would be of importance for frequency of drug administration is the terminal half-life in the brain. It is dependent on the relation between the $k_{out}$ (elimination rate constant from brain) and $k_{el}$ (elimination rate constant from systemic circulation). A $k_{out} < k_{el}$ will result in longer terminal half-life in brain than in blood (Hammarlund-Udenaes et al., 1997). A $k_{out}$ in turn is determined by the efflux clearance together with the brain volume of distribution. It has been shown that several drugs have longer half-lives in the brain compared with that in blood (Xie et al., 2000; Tunblad et al., 2003; Liu et al., 2005; Tunblad et al., 2005; Yang et al., 2005).

Techniques to Study Brain Delivery of Drugs

The in vivo methods to study the distribution of drugs to the brain ranges from simple brain tissue sampling to complex techniques like PET (de Lange et al., 1999; Golden and Pollack, 2003; Smith, 2003; Bickel, 2005). The methods used in this thesis are described in detail below.

Brain Tissue Sampling

Brain tissue sampling is a very simple technique that involves direct sampling of the brain tissue. It provides a time point estimate of total concentration of a compound in the whole brain in one animal. The concentration measure includes free intracellular and extracellular concentrations as well as the bound drug. In this approach, the compound is administered systemically and the animal is sacrificed at a specified time post-dose for the collection of brain tissue and blood. Either steady state concentration or AUC in the brain should be compared to the same in the plasma. Techniques like brain uptake index (Oldendorf, 1970), in situ perfusion (Takasato et al., 1984), iv injection technique (Ohno et al., 1978) and brain efflux index (Kakee et al., 1996) also involve sampling of the whole brain tissue.
Microdialysis

The microdialysis technique has gained increasing popularity over the last decade for studying drug distribution to the CNS (Elmquist and Sawchuk, 1997; de Lange et al., 1999; Hammarlund-Udenaes, 2000; Deguchi and Morimoto, 2001). With microdialysis, the concentration of unbound drug in the brain ISF can be measured continuously over time in vivo within one animal. This can then be compared with the blood concentration time profile measured with microdialysis or direct sampling, which contributes significant body of knowledge on brain delivery of drugs. In this technique a probe containing a semipermeable membrane is inserted into the tissue of interest and is continuously perfused with physiological solution called perfusate. Depending on the cut-off of the membrane only solutes that are smaller than the pores of the membrane are allowed to pass through the semipermeable membrane (Figure 3).

![Figure 3. Microdialysis probe. The perfusate enters through the inner cannula to the tip of the probe. Thereafter it flows upwards between the inner cannula and the membrane, where dialysis takes place (from Ungerstedt, 1991).](image)

The fluid leaving the probe, the dialysate, is collected at predetermined intervals. Since there is continuous flow of perfusate through the probe, concentration of the molecule in the dialysate is lower than in the tissue. The fraction of the tissue concentration recovered is referred as the relative recovery or simply the recovery. The factors which influence the recovery include physicochemical properties of the solute, probe geometry, membrane material, dialysate flow rate and tissue of interest (Bungay et al., 1990; de Lange et al., 2000).

The methods available for the estimation of in vivo recovery are no net flux (Lonnroth et al., 1987), dynamic no net flux (Olson and Justice, 1993), retrodialysis by drug (Bouw and Hammarlund-Udenaes, 1998), and retrodialysis by calibrator.
BBB and cetirizine enantiomers

(Wang et al., 1993). Retrodialysis by drug was used in Paper III in this thesis. The retrodialysis method is based on the assumption that the diffusion of a molecule across a semipermeable membrane is independent of the direction of the movement, therefore a molecule can be added to the perfusate and the disappearance rate through the membrane gives an estimate of in vivo recovery. This assumption has been tested in vitro (Zhao et al., 1995). A series of drug molecules which exist as neutral, cation or anion at physiological pH were used and it was demonstrated that the recovery of the molecules for given probes under the conditions tested was independent of the direction of movement. However, the drug investigated in this thesis showed differences in the recovery in the two directions. Different membranes investigated (PC, CUP, PES and PAES) gave similar results. The bovine serum albumin, BSA 0.5 % w/v was then added to the perfusate resulting in equal recovery values in both the directions. BSA also resulted in faster equilibration across the probe membrane. The use of BSA in microdialysis literature for increasing the recovery of molecules has been reported (Carneheim and Stahle, 1991; Trickler and Miller, 2003). Although the exact mechanism(s) of the effect observed in this thesis was not investigated, BSA is most likely decreasing the interaction of the drug with materials used in the microdialysis setup.

A concern with the microdialysis technique when studying BBB transport is the local tissue damage caused by probe insertion. However, by understanding the time-course of the changes in the tissue properties the experiment could be performed under optimal conditions (Tossman and Ungerstedt, 1986; Benveniste and Diemer, 1987; de Lange et al., 1995; Westergren et al., 1995). Another crucial factor to consider is the probe implantation procedure.

Stereoselectivity

Many drug molecules have an asymmetric center in their structure and therefore exist as a mixture of R and S enantiomers. In biological system, three dimensional structure of proteins make them highly stereo-specific and their interaction with the enantiomers of a drug could differ leading to stereoselective pharmacokinetics and pharmacodynamics (Lapicque et al., 1993; Mehvar et al., 2002; Brocks and Mehvar, 2003). Levocetirizine has a 30-fold higher affinity for H1 receptors than dextrocetirizine (Gillard et al., 2002). Disopyramide and verapamil have stereoselective binding to serum proteins and tissue phosphatidylserine (Hanada et al., 1998). Disopyramide also undergoes stereoselective metabolism because of difference in the affinity of its enantiomers for binding to the metabolizing enzymes (Echizen et al., 1994). Transport proteins located in organs with excretory function (liver and kidney) and barrier function (intestine, BBB, maternal-fetus barrier and blood-testis barrier) may also lead to stereoselective absorption, distribution and excretion of drugs. The pharmacokinetics of CZE in blood is known to be stereoselective in humans (Baltes et al., 2001). R-CZE has a longer half-life (7.8 vs. 5.5 h), a lower apparent clearance (41.7 vs. 90.6 ml/min) and a smaller apparent
volume of distribution (0.41 vs. 0.60 l/kg) than S-CZE. Choi et al. showed that the pharmacokinetics of the CZE enantiomers was not stereoselective in rats (Choi et al., 2000).

**Stereoselective Transport Across the BBB**

Stereoselective transport across the BBB has been demonstrated *in vitro* using bovine brain microvessel and immortalised rat brain capillary endothelial cell lines (Rochat et al., 1999; Pham et al., 2000). In *in vivo* studies total concentrations of enantiomers in the brain are often compared to that in blood or plasma (Baudry et al., 1997). However, in order to make conclusions regarding a possible stereoselective transport process differentiation needs to be made between all the factors affecting this distribution i.e. binding in blood, brain tissue and transport process per se as discussed above. The $K_{p,uu}$ measuring the transport across the BBB thus would indicate stereoselectivity in the BBB transport.

For apomorphine, comparison of total plasma and total brain concentrations indicated no difference in the brain distribution of the enantiomers. However, the ratio of unbound concentrations in plasma and brain ISF of R-apomorphine was higher than S-apomorphine indicating stereoselective uptake at the BBB. The stereoselective transport process resulted in higher unbound concentrations of R-apomorphine in the brain ISF (Sam et al., 1997). On the contrary, stereoselective transport of gacyclidine enantiomers across the BBB in rats resulted in similar exposure of its enantiomers (Hoizey et al., 2000). This indicates that process other than transport across the BBB must be stereoselective. From a pharmacodynamic point of view it is the unbound concentration in the brain ISF which is important given that the target receptors are located on the cell membrane.

The role of a specific transporter has successfully been studied using knockout mice. Stereoselective efflux mediated by P-gp at the BBB was demonstrated for methadone enantiomers using Abcb1a+/+ (wild type) and Abcb1a––/– (knockout) mice (Wang et al., 2004). This was evident by the different ratios of brain (R)- and (S)-methadone in the Abcb1a+/+ than those of the Abcb1a+/+ mice (15.3 versus 23.5) and by the different ratios of brain to plasma concentrations for (R)- and (S)-methadone in the Abcb1a––/– than those of the Abcb1a+/+ mice (12.8 versus 48.5). The assumption here is that the tissue and plasma binding is the same in knockout and wild type animals.

In another study, mefloquine enantiomers were compared in mice with and without the administration of the P-gp inhibitor elacridar (Barraud de Lagerie et al., 2004). It was shown that mefloquine enantiomers undergo efflux at the BBB in a stereoselective manner. The drawback with the measurement of only total concentrations is that the differences in plasma protein binding and the distributional aspect of drugs within the brain cannot be addressed.
Measurement of CNS Side-effects of $\text{H}_1$-antihistamines

Investigation of central side-effects caused by antihistamines can be performed in humans by objective psychometric tests and subjective rating scales (Hindmarch and Shamsi, 1999). Using various antihistamines at therapeutic doses, Yanai et al demonstrated the existence of a correlation between impairment of cognitive performance and $\text{H}_1$ receptor occupancy (Yanai et al., 1999). Sedative effects or cognitive impairment observed in humans are difficult to observe in animal species. However, receptor occupancy determination could be easily assessed in animal species using ex-vivo binding assay at central sites. In this thesis, $\text{H}_1$ receptor occupancy in the brain was used as a surrogate biomarker of sedation. The guinea pig was selected as the animal model as they have high brain concentrations of $\text{H}_1$ receptors compared to rats (Hill and Young, 1980) and are often used in antihistamine work. Ex-vivo $\text{H}_1$ receptor occupancy is often carried out at the time of maximum plasma concentration (Wiech and Martin, 1982; Snowman and Snyder, 1990; Kreutner et al., 2000; Yakuo et al., 2001). However, for some drugs the plasma concentrations are directly correlated to the brain $\text{H}_1$ receptor occupancy while for others no correlation could be observed (Tagawa et al., 2001). The brain pharmacokinetics may differ significantly from plasma pharmacokinetics because of the BBB transport (Xie et al., 2000; Tunblad et al., 2003; Tunblad et al., 2005) which in turn could contribute to delayed CNS effects (Welty et al., 1993; Bouw et al., 2000; Bouw et al., 2001; Chenel et al., 2004). The knowledge of time course of the CNS effect or side effects and its relationship with concentrations is thus important information for optimization of therapeutic regimens of these drugs.
Aims of the Thesis

The general aim of this thesis was to study the role of the BBB in the pharmacokinetics and pharmacodynamics of cetirizine enantiomers in the brain.

The Specific aims were

- to develop and validate a quantitative method for the determination of CZE enantiomers in guinea pig plasma, brain tissue and microdialysis samples using LC/MS/MS
- to characterize the pharmacokinetics of CZE enantiomers in the guinea pig and to study the differences in the protein binding both in guinea pig and human plasma
- to quantify the transport of CZE enantiomers across the BBB and to investigate factors that could lead to stereoselective brain distribution
- to study the time course of H1 receptor occupancy of levocetirizine in the brain and the periphery in relation to the brain and plasma concentrations of the drug and characterize the influence of the BBB in any delays observed in central H1 receptor occupancy
Materials and Methods

Animals
Male Dunkin Hartley guinea pigs weighing 350-550 gram (Mollegaard, Denmark and Charles River, France) were acclimatized for at least 5 days prior to the surgery. They were group housed at 22°C under controlled humidity and a 12 h light-dark cycle. Standard diet and water were provided ad libitum. The protocols were approved by the Animal Ethics Committee of Uppsala University (C218/1; Paper I to IV) and UCB (orgisol-GP; Paper IV).

Surgical Procedure
The guinea pigs in Papers II, III and IV were anaesthetized by inhalation of Isoflurane® (2.5 % balanced with 1.5 l/min oxygen and 1.5 l/min nitrous oxide) and 0.25 ml of Dormicum® (midazolam 5 mg/ml) intraperitoneally. They were placed on a heating pad to maintain the body temperature at 38°C during surgery. FEP tubings were inserted into the left jugular vein for drug administration and into the left common carotid artery for blood sampling. In order to avoid clotting, the catheters were filled with heparinized saline solution (100 IU/ml). In Paper III, blood and brain probes were inserted in the guinea pig. The blood probe (CMA/20, Polycarbonate, 20 kD cutoff, 10 mm) was inserted into the right jugular vein and sutured to the pectoral muscle. A stereotaxic instrument (Davis Kopf Instruments, Tujunga, USA) was used for the insertion of a brain probe (CMA/12, Polycarbonate, 20 kD cutoff, 3 mm) at the coordinates -1.0 mm lateral and 1.1 mm anterior to the bregma point and 3.2 mm ventral to the surface of the brain. A piece of FEP tubing was looped subcutaneously on the back of the guinea pig to the posterior surface of the neck, to let the perfusion solution adjust to body temperature before entering the brain probe. The ends of the catheters were passed subcutaneously and gathered in a plastic cup that was sutured to the posterior surface of the neck. The guinea pigs were placed in individual CMA/120 cages for freely moving animals with free access to water and food, where they recovered for 24 h prior to the experiments.
Recovery

Probe Characterization *in vitro*

In Paper III each microdialysis probe was calibrated *in vivo* using the method of retrodialysis by drug (Bouw and Hammarlund-Udenaes, 1998). This method assumes that the recovery is the same in both directions across the probe membrane. This was verified *in vitro* before performing *in vivo* experiments.

For direct dialysis (gain of analyte from external medium to probe) a CMA/12 probe was continuously perfused with blank perfusate at a flow rate of 0.5 μl/min and dialysate were collected every 15 min (C_{out}). During the first 30 min the probe was placed in a tube containing blank Ringer, then in Ringer solution containing CZE (50 ng/ml, C_{m}) for 90 min and then back in blank Ringer solution for 120 min washout period. For retrodialysis (loss of analyte from probe to external medium), the same probe was perfused with Ringer solution containing CZE (50 ng/ml, C_{m}) for 90 min followed by blank Ringer solution for 120 min. The probe was placed in blank Ringer solution during the retrodialysis experiment.

The external medium was stirred constantly. The experiments were performed at 37°C in triplicates. The perfusion fluids investigated were Ringer solution and Ringer solution with 0.5 % w/v BSA (Initial fractionation by heat shock; Sigma Chemical Co., St Louis, MO, U.S.A.). The Ringer solution used in the study consisted of 33 mg KCl, 330 mg CaCl2·H2O, 8.6 gram NaCl, sterilized water for injection Ph. Eur. ad 1000 ml, pH 6 (Hospital Pharmacy, Uppsala, Sweden). The relative recovery of CZE enantiomers *in vitro* by the direct dialysis method was calculated by the formula, Recovery = C_{out}/C_{m} and the retrodialysis recovery was determined by the formula, Recovery = (C_{in}-C_{out})/C_{in}.

Probe Calibration *in vivo*

Ringer solution containing 0.5 % BSA was used as the perfusate in Paper III. The probes were perfused with a blank Ringer with BSA for 60 min at a flow rate of 0.5 μl/min to stabilize the system and to obtain blank samples for the chemical analysis. During the retrodialysis period, the perfusion solution for the blood probe and the brain probe contained 50 and 25 ng/ml CZE, respectively. In total five samples were collected in fractions of 15 min during this period. The perfusion fluid was subsequently changed to blank Ringer with BSA. A washout period of 90 min was allowed to clean the system. The relative recovery of CZE was estimated in each guinea pig and a mean value of at least 3 estimations during the retrodialysis period was used to calculate the unbound concentrations of S- and R-CZE in brain interstitial fluid (ISF) and plasma from the dialysate concentrations.
Study Design

In Paper II CZE was administered as a 10 min constant rate i.v. infusion. Three dose levels 1 (n=5), 2 (n=6) and 4 mg/kg (n=5) were administered to detect a possible non-linear behavior in the pharmacokinetics of CZE enantiomers. Blood was collected at a pre-defined schedule until 29 h post-dosing. Plasma was separated by centrifugation of blood at 10 000 rpm for 5 min.

The experimental setup used in Paper III is shown in Figure 4. The experiment was started 24 h after the surgery. After the stabilization period of 60 min, the probe calibration period of 75 min and washout period of 90 min, CZE was administered as a 60 min constant rate infusion, resulting in a total dose of 2.7 mg/kg. Blood and brain ISF were continuously sampled by microdialysis. Dialysate fractions were collected every 15 min during the infusion and 1 h post-infusion and then every 30 min for the next 4 h. Arterial blood samples were collected at a pre-defined schedule and the plasma was separated. The guinea pig was decapitated at the end of the experiment and the brain was divided into the right and left hemispheres, weighed and immediately frozen at -20°C until analysis.

In Paper IV levocetirizine was administered orally at doses of 0.1 and 1 mg/kg to conscious guinea pigs at time zero. The guinea pigs were sacrificed at 1, 2, 4, 8 and 16 h. Blood, cerebellum and ileum were sampled and the plasma was separated from blood. One half of the cerebellum was used to study the ex-vivo H₁ receptor occupancy and the other half was used to measure concentrations of levocetirizine. The ileum was collected in order to estimate peripheral H₁ receptor occupancy. Four animals were included at each time point. One saline control group was included to obtain estimates of 0 % H₁ receptor occupancy. One chlorpheniramine group was included as a positive control to define the maximal H₁ receptor occupancy. Chlorpheniramine (2 mg/kg) was administered intravenously and blood, cerebellum and ileum were sampled at 1 h after dosing. Another group of animals was included to characterize the full plasma concentration-time profile after oral administration of levocetirizine. The guinea pigs with catheters inserted into the left common carotid artery for blood sampling were administered levocetirizine orally at the doses of 0.1 (n=3) and 1 mg/kg (n=4) and the blood was collected at a pre-defined schedule and
the plasma was separated. Samples from all the studies were stored at -20°C pending analysis.

**Plasma Protein Binding**

Equilibrium dialysis was used to assess the protein binding of CZE enantiomers in guinea pig and human plasma *in vitro* in Paper II. Guinea pig blank plasma was spiked with CZE stock solution to obtain final concentrations of 25, 250 and 2500 ng/ml of each enantiomer. Five hundred μl of spiked plasma, adjusted to physiological pH was dialyzed against the same volume of sodium phosphate buffer (10 mM, pH 7.4) for 5 h in an atmosphere of 5 % CO₂ in air. Dialysis was performed in triplicates at 37°C under constant stirring (20 rpm). At equilibrium, samples from both plasma and buffer sides were collected. Human plasma was spiked with 500 ng/ml of CZE and dialysis was performed for a period of 3 h.

**Ex vivo H₁ Receptor Binding in Brain**

In Paper IV central H₁ receptor occupancy was determined using displacement of [³H]-mepyramine binding to the cerebellum. Half cerebellum, from saline, chlorpheniramine or levocetirizine treated guinea pigs, were homogenized in 250 μl of a 20 mM Tris HCl buffer (pH 7.4) containing 250 mM sucrose and stored in liquid nitrogen. Subsequently, prewarmed homogenates (10 μl) were incubated for 1 min at 25°C in a 490 μl of a 50 mM Tris HCl buffer (pH 7.4) containing 2 mM MgCl₂ and 3 nM [³H]-mepyramine. After the incubation period, the receptor bound radioligand was separated from the free ligand by vacuum filtration of samples over GF/C glass fiber filters (Whatman, VEL, Belgium) and radioactivity trapped onto the filter was determined in a liquid scintillation counter (30-40 % efficiency). Nonspecific binding (NSB) of [³H]-mepyramine to the homogenate was measured in the presence of 10 μM CZE. Receptor specific binding (RSB) was obtained by subtracting the NSB from the total binding observed in the absence of CZE (B₀). For each sample, two B₀ and two NSB were obtained. The specific binding was also corrected for the amount of proteins in each sample. The percentage of H₁ receptor occupied was expressed as

\[
\% \text{ H₁ Receptor Occupancy} = \frac{\text{RSB}_{\text{treatment}} - \text{RSB}_{\text{saline}}}{\text{RSB}_{\text{saline}}} \times 100
\]  

(7)

**Ex vivo H₁ Receptor Binding in Ileum**

Peripheral H₁ receptor occupancy was studied using the shift of histamine concentration-response curves in segments of ileum obtained from the same guinea pigs. Sections of ileum were mounted in 20 ml organ baths filled with Tyrode solution.
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The bathing solution was maintained at 37°C and gassed with 95 % O₂–5 % CO₂. Tissues were allowed to equilibrate for a period of 20 min under a resting tension of 1 g. Isometric contractions were measured by force-displacement transducers coupled to an IOX computer system (EMKA Technologies, Paris, France). At the end of the 20 min period of stabilization, a cumulative concentration-response curve to histamine was elicited.

Agonist activity of histamine was determined by the calculation of pD₂ values (Van Rossum et al., 1963). The concentration of histamine inducing half the maximal response (pD₂) and the maximal response of each concentration-response curve (E_max) were calculated by an iterative computer software (XLfit, ID Business Solutions, United Kingdom or Prism, GraphPad software, San Diego, USA) fitting the experimental data to the four parameter logistic equation:

\[
Y = A + \frac{B - A}{1 + \left(\frac{10^{C}}{10^{X}}\right)^{D}}
\]

where \(Y\) is the observed effect, \(A\) is minimum \(Y\), \(B\) is maximum \(Y\), \(C\) is -pD₂, \(D\) is the slope factor and \(X\) is the log of the molar concentration of histamine.

In the presence of levocetirizine, a higher concentration of histamine will be required to produce the same effect and the histamine concentration response curve on the guinea pig ileum will shift to the right. The shift between the saline and levocetirizine treated ileum curve was estimated from the ratio of histamine concentrations required to produce a half maximal response to histamine in saline or levocetirizine treated animals. Since levocetirizine is a competitive histamine \(H_1\) antagonist as previously described (Christophe et al., 2003), a theoretical calculation based on the shift between these curves allows an estimation of the peripheral receptor occupancy, for example, a two-fold shift would correspond to 50 % of the receptors being occupied by the antagonist.

Chemical Assay

Sensitive enantioselective liquid chromatographic assays using tandem mass spectrometry detection (LC/MS/MS) were developed and validated for the determination of S-CZE and R-CZE in guinea pig plasma, brain tissue and microdialysis samples (Paper I).

The chromatographic separation of the CZE enantiomers was achieved by a Chiral-AGP column (α-acid glycoprotein, 150×4.0 mm, ChromTech, Hägersten, Sweden) tandem with a triple quadropole mass spectrometer (Quattro Ultima; Waters, Manchester, UK). The triple-quadropole mass spectrometer was used in the positive electrospray mode (ESI). The transitions that gave the most intense peaks were m/z 389.1>200.9 for CZE and 396.1>276.1 for internal standard (IS; ucb 20028; Figure 5b).
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Figure 5. The chemical structures of (a) cetirizine: \(2-[4-[(4\text{-Chlorophenyl})\text{phenylmethyl}]\text{1-piperazineyl}]\text{ethoxy}\text{acetic acid chlorhydrate}\) and (b) Internal standard (ucb 20028) \(2-[2\text{-}(4\text{-Benzhydrylidenepiperidin-1-yl})\text{ethoxy}]-\text{ethoxy}\text{-acetic acid chlorhydrate}\). The star represents the chiral center.

Table 1. Mass spectrometer instrument settings.

<table>
<thead>
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<th>Value</th>
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</thead>
<tbody>
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</tr>
<tr>
<td>Source temperature (°C)</td>
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</tr>
<tr>
<td>Cone gas flow (l/h)</td>
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<td>Collision gas pressure (Torr)</td>
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<tr>
<td>Inter scan delay (s)</td>
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</tbody>
</table>

The instrument settings that gave the most intense peaks were selected (Table 1). The resolution was set at 0.9 u at half the height for Q1 and Q3. The collision energy was set to 20 eV for CZE and 25 eV for IS. The separation of R and S-CZE is shown in Figure 6 a and b.

For the plasma method, a standard curve consisting of ten standard samples containing each enantiomer in the range of 0.25-5000 ng/ml was prepared. Three quality control, QC: low (QCL, 0.75 ng/ml) medium (QCM, 250 ng/ml) and high (QCH, 3710 ng/ml) were prepared. The samples were prepared by the precipitation of 50 l plasma with 100 l acetonitrile (ACN), containing IS (100 ng/ml). Fifty l of the supernatant was evaporated and the residue was dissolved in 500 l mobile phase and 50 l was injected on to the LC-MS/MS system. The mobile phase consisted of 6.5 % ACN in 10 mM ammonium acetate, pH 7.0 (adjusted with 1 % ammonia) pumped at a flow-rate of 0.9 ml/min. The column was maintained
at 30°C in a water bath. The assay was linear over the range 0.25 to 5000 ng/ml for both S-CZE and R-CZE. Precision (expressed as coefficient of variation) and accuracy for the lower limit of quantification, LLOQ (0.25 ng/ml) was 13 % and 115 %, respectively. Plasma samples from Paper II and III were analyzed by this method.

Figure 6. Chromatograms of (a) a spiked guinea pig plasma sample containing 125 ng/ml of S- and R-cetirizine, and 100 ng/ml of the IS (b) a spiked Ringer-BSA solution sample containing 25 ng/ml of S- and R-cetirizine, and 2 ng/ml of the IS and (c) a spiked guinea pig plasma sample containing 500 ng/ml of R-cetirizine, and 100 ng/ml of the IS.
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For the brain method, the blank brain sample was prepared by homogenizing blank brain tissue with a 4-fold volume (w/v) of saline. The standard samples were prepared by spiking blank brain tissue samples with the stock CZE solutions before homogenization. The standard curve for guinea pig brain composed of 7 samples containing each enantiomer in the range of 2.5-250 ng/g brain. Three QC samples (concentrations 12.5, 75 and 188 ng/g brain) were prepared. Fifty μl of the brain homogenate was sampled and processed as in the plasma method but with the following modifications: the concentration of IS used was 5 ng/ml, the residue was dissolved in 1000 μl mobile phase and 40 μl was injected. The assay was linear over the range 2.5 to 250 ng/g brain for both S-CZE and R-CZE. Precision and accuracy for LLOQ (2.5 ng/g) was 10 % and 104 %, respectively. Brain samples from Paper III were analyzed by this method.

For the Ringer method, BSA was soaked in Ringer solution overnight and the volume was adjusted the next day to give the blank 0.5 % BSA in Ringer solution. This solution was then spiked with CZE stock solution to give eight standard samples in the range of 0.25-50 ng/ml for each enantiomer. Three QC samples (concentrations 0.38, 7.5 and 38 ng/ml) were prepared. For the Ringer method, the column was maintained at room temperature and 5.5 % ACN was used in the mobile phase. A switch was used after the analytical column to avoid salt overloading in the mass spectrometer. After sample injection, the flow of the mobile phase from the column was diverted to waste until 6.5 min. The BSA in standard and QC samples (8 μl) were precipitated with 100 μl ACN containing IS (2 ng/ml) as described above. The supernatant (100 μl) was transferred to an Eppendorf tube and evaporated. The assay was linear over the range of 0.25-50 ng/ml for both the enantiomers. Precision and accuracy for LLOQ (0.25 ng/ml) was 13 % and 104 %, respectively. The microdialysis samples from Paper III were analyzed by this method.

To analyze the phosphate buffer samples from in vitro protein binding study, 6 μl of the buffer samples were directly injected on to the column. The column was maintained at room temperature. The mobile phase consisted of 10 mM ammonium acetate, pH 7.0 (adjusted with 1 % ammonia) and 5.5 % ACN. The standard curve was linear over the range of 0.5 to 250 ng/ml for both the enantiomers. Precision and accuracy for LLOQ (0.5 ng/ml) was 14 % and 113 %, respectively.

In Paper IV, plasma and cerebellum samples were analyzed for the contents of levocetirizine using a Zorbax SB-CN (150×4.6 mm, Agilent Technologies, Wilmington, DE, USA) column tandem with a triple quadropole mass spectrometer (Figure 6c). The instrument settings are the same as shown in Table 1 with the following differences: The desolvation and cone gas flow was set to 700 l/h and 250 l/h, respectively and capillary voltage used was 2.8 kV. The mobile phase consisted of 10 mM ammonium acetate buffer at pH 7.0 and 35 % ACN. The microdialysis samples from Paper III were analyzed by this method.
The assay was linear over the range of 0.1-100 ng/g brain. Precision and accuracy for the LLOQ (0.1 ng/g brain) was 7.3 % and 109 %, respectively. For plasma samples, the dried sample was dissolved in 250 µl mobile phase. The standard curve was splitted into two, a low (0.5-250 ng/ml) and a high (250 - 2500 ng/ml) curve. The precision and accuracy for the LLOQ (0.5 ng/ml) was 10 % and 100.3 %, respectively.

Data Analysis

Modeling

In Paper II, the concentration-time data of CZE enantiomers was analyzed by nonlinear mixed effect modeling using the computer program NONMEM version VI (Beal and Sheiner, 1994). The models were developed using simultaneous estimation of fixed effects that characterize the typical individual and random effects that quantify variability (inter-individual variability (IIV) and residual variability) component.

An exponential variance model was used to describe the IIV in the parameters.

\[ P_i = P * \exp(\eta_i) \]  

\( P \) denotes the individual parameter prediction, \( P \) the typical value of the parameter and \( \eta_i \) the individual random effect that accounts for the difference between the typical predicted value and the individual estimate. The \( \eta_i \) is a symmetrically distributed zero mean variable with variance of \( \omega^2 \).

To describe residual variability an additive or proportional error model or combination of the two with the original or log-transformed data were considered e.g.:

\[ \ln C_{ij} = \ln C'_{ij} + \varepsilon \]  

where \( C_{ij} \) and \( C'_{ij} \) are the observed and predicted CZE enantiomer concentrations for the \( j \)th individual at time \( i \), respectively, and \( \varepsilon \) is the additive error (with zero mean and variance \( \sigma^2 \)). The residual error is used to account for unexplained difference in prediction and observed individual concentrations.

The estimations were made using the first order conditional estimation method (FOCE). One-, two-, and three-compartment models with first order elimination from the central compartment were considered and specified to NONMEM by ADVAN5. The two enantiomers were modeled simultaneously with a common epsilon to account for potential covariance between the measurements of the two enantiomers. To estimate the differences in the pharmacokinetic parameters of the CZE enantiomers, all the parameters of S-CZE were parameterized as
\[ P_{i,S} = P_{i,R} * MF_{1i} \] (11)

\( P_{i,S} \) denotes the individual parameter prediction for S-CZE, \( P_{i,R} \) is the individual parameter prediction for R-CZE and \( MF_{1i} \) is the individual multiplication factor describing the difference between all the pharmacokinetic parameters estimated for the two enantiomers. To check for further differences not explained by \( MF_{1i} \), another factor \( MF_{2} \) was included. The need for \( MF_{2} \) was evaluated on each parameter.

Models were assessed based on graphical analysis using Xpose 3.1 (Jonsson and Karlsson, 1999) and objective function value (OFV) from NONMEM. To distinguish between two nested models a drop in the OFV of 3.84 was required, which corresponds to a nominal value of \( p < 0.05 \).

**Noncompartmental Analysis**

In Paper III, brain distribution of CZE enantiomers was compared using the parameters \( K_{p} \) (Equation 1), \( K_{p,u} \) (Equation 3) and \( K_{p,uu} \) (Equation 5). In Paper IV, the \( K_{p} \) value for levocetirizine was also calculated. All calculations in this paper were based on the mean concentration-time data; each data point was the mean of four animals. Therefore, the pharmacokinetic parameter estimates are expressed only as mean values.

The areas under the concentration-time curves \( AUC_{t-\infty} \) were expressed as the sum of area under the corresponding concentration versus time curve until the last observation (\( AUC_{0-t} \)) and the residual area (\( AUC_{t-\infty} \)). The \( AUC_{0-t} \) was calculated by the linear trapezoidal method. The \( AUC_{t-\infty} \) was determined as the ratio of the concentration at the last time point to respective terminal rate constants. In Paper III, the total concentration-time profile in brain was generated using the unbound concentration-time profile in brain ISF obtained from the microdialysis experiment and the fraction unbound in brain ISF at 360 min after the infusion \( f_{u,brISF,360} \) with the assumption that the fraction unbound of CZE enantiomers in brain was constant with time.

To estimate binding of CZE enantiomers within the brain the \( V_{u,br} \) at 360 min was calculated. In Equation 6, instead of \( V_{bl} \) and \( C_{bl} \) plasma volume per gram brain and total plasma concentrations of CZE enantiomers were used. The plasma content in the brain of the guinea pig has been estimated as 11.5 \( \mu l \) per gram brain (Bosse and Wassermann, 1970). The assumption is that CZE enantiomers are not or only poorly associated with blood cells in guinea pigs (Benedetti et al., 2001). \( A_{br} \) was determined in the whole brain sample taken at 360 min.

The terminal half-life \( (t_{1/2}) \) in plasma and brain was determined from the terminal rate constant, which was itself obtained from log-linear regression of the terminal phase of the concentration-time curve (Paper III and IV).

In Paper III, individual plasma clearance (CL) and apparent volume of distribution (V) were computed as \( CL = \frac{Dose_{iv}}{AUC_{tot,pl,0-\infty}} \) and \( V = \frac{CL}{\lambda} \) respectively, where \( \lambda \) is the terminal rate constant. The unbound clearance (\( CL_{u} \)) and volume of
distribution ($V_d$) in plasma were also calculated as described above using the unbound concentration time curve in plasma obtained from microdialysis experiment.

In Paper II, $f_{u,pl}$ determined *in vitro* was calculated by the ratio of drug concentrations measured in the buffer and plasma after dialysis. In Paper III $f_{u,pl}$ *in vivo* was estimated as the ratio of unbound plasma AUC obtained from microdialysis experiment and total plasma AUC obtained from regular arterial blood sampling.

**Statistics**

In Paper III, CZE enantiomers were compared for brain distribution and blood pharmacokinetic parameters using Exact Wilcoxon’s signed-rank test (S-plus® 6.1 for Windows, Insightful Corp., Seattle, USA) as the sample number was too low to assume normal distribution of the population parameters. However, the parameters $K_{pu}$, $K_{puu}$, $K_{puu}$ and $f_{u,pl}$ for S and R-CZE were compared using paired t-test as proportions are normally distributed. A p-value of ≤ 0.05 was considered to be statistically significant.
Results and Discussion

Stereoselective Plasma Pharmacokinetics of Cetirizine (Paper II and III)

The plasma pharmacokinetics of CZE was stereoselective in guinea pigs (Paper II and III). The total plasma concentrations of R-CZE was higher than S-CZE (Figure 7 and 8). In Paper II, the data was best described by a three compartment pharmacokinetic model. The model was parameterized as clearances and volumes of distribution. The CL of R- and S-CZE was 1.2 (RSE=7.5 %) and 2.7 ml/min (RSE=7.5 %), respectively in Paper II and 1.2 ± 0.2 and 3.2 ± 0.7 ml/min, respectively in Paper III. The volume of distribution at steady state, $V_{ss}$ is equal to the sum of the apparent volumes of the central and peripheral compartments (Gibaldi and Perrier, 1982). Thus, the average value of $V_{ss}$ was 449 ml for R-CZE and 979 ml for S-CZE (Paper II). In Paper III, $V$ calculated from CL and terminal rate constant was 268 ± 98 for R-CZE and 626 ± 196 for S-CZE. The discrepancy between the volumes of distribution in Paper II and III could be due to over-prediction of terminal rate constant in Paper III.

Figure 7. Observed plasma concentration-time profile of the cetirizine enantiomers after a 10 min short i.v. infusion of racemic cetirizine at doses 1 (n=5), 2 (n=6) and 4 mg/kg (n=5) in guinea pigs.
CZE is a low extraction ratio drug based on the low plasma CL of CZE enantiomers as compared to the hepatic (46 ml/min) and renal blood flow (36 ml/min) in guinea pigs (Gabrielsson and Weiner, 2000). For a low extraction ratio drug both hepatic and renal clearance depends on $f_{ub}$ (Rowland, 3rd Edition). In Paper II differences in most of the pharmacokinetic parameters of the CZE enantiomers could be explained by one common factor (MF1) (Table 2). The typical value of CL for S-CZE was 2.26 (MF1) times higher than that for R-CZE. In the protein binding experiment the $f_{ub}$ for S-CZE in the was 2.1-2.3 times higher than R-CZE. This indicates that protein binding is the primary factor affecting the clearance of the CZE enantiomers. The volume of distribution for the peripheral compartment of S-CZE was also 2.26 times higher than that for R-CZE. However, the central volume of distribution and one of the inter-compartmental clearances were 1.72 (MF1*MF2) times higher than R-CZE (Table 2).

**Table 2.** Population pharmacokinetics parameter estimates of the CZE enantiomers for the typical individual after administration of racemic CZE (1, 2, and 4 mg/kg) as a 10 min constant rate infusion. The estimates of inter-individual variability (IIV) and residual error are given as coefficient of variation (%). The estimate of precision of the parameter estimate is given in parenthesis as relative standard error (RSE %). (From Paper II)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Population average</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R-CZE</td>
</tr>
<tr>
<td>CL (ml/min)</td>
<td>1.2 (7.5)</td>
</tr>
<tr>
<td>$Q_{p1}$ (ml/min)</td>
<td>15.5 (15)</td>
</tr>
<tr>
<td>$Q_{p2}$ (ml/min)</td>
<td>0.45 (12)</td>
</tr>
<tr>
<td>$V_c$ (ml)</td>
<td>66.6 (12)</td>
</tr>
<tr>
<td>$V_{p1}$ (ml)</td>
<td>141 (9.7)</td>
</tr>
<tr>
<td>$V_{p2}$ (ml)</td>
<td>249 (15)</td>
</tr>
<tr>
<td>MF1</td>
<td>2.26 (3.4)</td>
</tr>
<tr>
<td>MF2</td>
<td>0.76 (2.3)</td>
</tr>
<tr>
<td>IIV CL</td>
<td>24 (37)</td>
</tr>
<tr>
<td>IIV $V_c$</td>
<td>38 (41)</td>
</tr>
<tr>
<td>IIV $V_{p1}$</td>
<td>42 (41)</td>
</tr>
<tr>
<td>IIV MF1</td>
<td>12 (37)</td>
</tr>
<tr>
<td>Residual error</td>
<td>R-CZE</td>
</tr>
<tr>
<td></td>
<td>R and S-CZE</td>
</tr>
</tbody>
</table>

Abbreviations: CL, clearance from central compartment, $Q_{p1}$ and $Q_{p2}$, inter-compartmental clearances, $V_c$, $V_{p1}$ and $V_{p2}$, volume of distribution of central, first and second peripheral compartments.

*The pharmacokinetic parameters for S-CZE were expressed as $P_s = P_r \cdot MF1$ for CL, $Q_{p1}$, $V_{p1}$ and $V_{p2}$ and as $P_s = P_r \cdot MF1 \cdot MF2$ for $Q_{p2}$ and $V_c$.

The volume of distribution of a drug depends on the relationship between the plasma protein binding and tissue binding. Therefore, minor involvement of factors other
than protein binding can not completely be ruled out. In Paper III, $V_d$ of the two enantiomers was the same whereas $CL_d$ was significantly different. This discrepancy in Paper II and III could be due differences in plasma protein binding. The $f_{upl}$ (0.50 and 0.15 for S- and R-CZE, respectively) determined *in vivo* using the microdialysis technique was higher as compared to the $f_{upl}$ (7-10 % for R-CZE and 16-21 % for S-CZE) determined by *in vitro* equilibrium dialysis. This difference could be due to altered albumin levels in guinea pigs that underwent microdialysis surgery. The albumin levels were lower (1.8-2.5 %) in the plasma of guinea pigs that underwent microdialysis surgery as compared to naïve animals (2.6-3.2 %).

Protein binding of CZE in human plasma was stereoselective with the $f_{upl}$ of 8 and 12 % for R- and S-CZE, respectively (Paper II). The $f_{upl}$ of 8 % for R-CZE in human plasma determined in the present study is comparable to the binding reported previously. Benedetti et al. showed that, at concentrations ranging from 0.2 to 1 $\mu$g/ml, the percentage bound of R-CZE was concentration independent (94.8-95.5 %) (Benedetti et al., 2001). Bree et al. also reported high binding (91.2 ± 0.66 %) of R-CZE to human plasma over the concentration range studied (0.21-4.98 $\mu$M) (Bree et al., 2002). Lower $f_{upl}$ of 5 % for R-CZE determined by Benedetti et al. could be because of the different technique used.

Baltes et al. compared the pharmacokinetics of R-CZE and S-CZE in 24 healthy volunteers by giving orally either R-CZE alone or racemic CZE (Baltes et al., 2001). The apparent volume of distribution of S-CZE was 1.5 times higher than R-CZE (0.41 vs. 0.60 l/kg) indicating that the difference in protein binding found in the present study could explain the difference in volume of distribution reported in humans. The apparent renal and non-renal clearance in humans was 61 and 28.9 ml/min, respectively, for S-CZE and 31 and 9.7 ml/min, respectively, for R-CZE (Baltes et al., 2001). The difference in renal clearance of the CZE enantiomers is approximately the same as the difference in $f_{upl}$ of the two enantiomers. It has been reported using human microsomes that the intrinsic clearance values describing the formation of metabolites were higher for S-CZE compared with R-CZE (Whomsley et al., 2003). Thus, the differences in non-renal clearance of the two enantiomers likely reflects differences both in metabolic intrinsic clearance and $f_{upl}$.

**Probe Characterization (Paper III)**

The *in vitro* characterization of the microdialysis probes showed that inclusion of 0.5 % BSA in Ringer was necessary for the recovery of the CZE enantiomers to be equal in both directions across the probe membrane. It was also contributing to a more rapid equilibration across the probe membrane. The *in vitro* recovery of S- and R-CZE determined by direct dialysis of the brain probes were 48.0 ± 4.6 % and 47.8 ± 4.9 % and by retridialysis were 44.9 ± 5.1 % and 44.4 ± 5.1 %, respectively (n.s.). The *in vivo* recovery of the CZE enantiomers with the brain probes were 18.5 ± 8.8 % and 18.8 ± 8.4 % for S- and R-CZE, respectively. With the blood probes the *in vivo* recoveries were 83.5 ± 11.7 % and 86 ± 10.5 %, respectively.
Brain Distribution of Cetirizine Enantiomers (Paper III and IV)

The unbound concentrations of CZE enantiomers in the brain were lower than in plasma (Figure 8), indicating low brain distribution of CZE. The total amount of the drug per gram brain and unbound brain ISF concentrations of S-CZE were 1.8 and 1.5 times higher than R-CZE (Table 3).

![Graph showing concentration-time profiles in blood and brain ISF of R-cetirizine and S-cetirizine](image)

**Figure 8.** Total-concentration time profile in blood and unbound-concentration time profiles (average ± SD) in blood and brain ISF of R-cetirizine and S-cetirizine obtained from regular blood sampling and microdialysis, respectively. The data was collected during and after a 1 h constant rate infusion of racemic cetirizine (2.7 mg/kg; n = 8). All unbound concentrations were corrected for recovery obtained by the retrodialysis by drug method before drug administration.

**Table 3.** Pharmacokinetic parameters in plasma and brain of S- and R-CZE in guinea pigs (n = 8) after intravenous constant rate infusion of racemic CZE (2.7 mg/kg). The results are presented as average ± SD. Statistical analysis was performed using Wilcoxon’s signed rank test. (From Paper III)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>S-cetirizine</th>
<th>R-cetirizine</th>
<th>Ratio S/R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl (ml/min/kg)</td>
<td>14.9 ± 2.10</td>
<td>18.8 ± 3.67</td>
<td>0.8**</td>
</tr>
<tr>
<td>V (ml/kg)</td>
<td>2808 ± 602</td>
<td>3028 ± 578</td>
<td>0.9</td>
</tr>
<tr>
<td>fump</td>
<td>0.50 ± 0.10</td>
<td>0.15 ± 0.03</td>
<td>3.3***</td>
</tr>
<tr>
<td>t1/2pl (h)</td>
<td>2.20 ± 0.45°</td>
<td>1.90 ± 0.45b</td>
<td>1.2**</td>
</tr>
<tr>
<td>t1/2br (h)</td>
<td>4.94 ± 1.40°</td>
<td>3.94 ± 1.63b</td>
<td>1.3*</td>
</tr>
<tr>
<td>AUCpl,0-360 (min*ng/ml)</td>
<td>68510 ± 9104</td>
<td>55545 ± 9576</td>
<td>1.2**</td>
</tr>
<tr>
<td>AUCbr,0-360 (min*ng/ml)</td>
<td>11435 ± 2843</td>
<td>7498 ± 1728</td>
<td>1.5**</td>
</tr>
<tr>
<td>A (ng/g brain)</td>
<td>45.9 ± 13.3</td>
<td>25.4 ± 9.0</td>
<td>1.8**</td>
</tr>
</tbody>
</table>

* p < 0.05, ** p < 0.01 and *** p < 0.001 for difference between the parameter for S-CZE and R-CZE
a (p < 0.01; statistical comparison of half life in plasma and brain for S-CZE)
b (p < 0.01; statistical comparison of half life in plasma and brain for R-CZE)
c Dose normalized AUC
d Total amount per g brain at 360 min after start of the infusion corrected for plasma volume in the brain.
The extent of brain distribution of the CZE enantiomers determined by $K_p$ might be interpreted as enantioselective, the value being $0.22 \pm 0.05$ for S-CZE and $0.04 \pm 0.01$ for R-CZE (Figure 9). As $K_p$ compares total brain concentration to total plasma concentration this measure includes all the three factors governing the distribution; protein binding in blood, binding to brain parenchymal cells and proteins, and transport across the BBB (Equation 2). A higher $K_p$ for S-CZE indicates that one or several of these factors are different. It has been shown recently for 23 clinically used central nervous system drugs that a 50-fold range in absolute $K_p$ values were largely determined by binding in brain and blood (Maurer et al., 2005). The $K_p$ for racemic CZE in mice has previously been reported to be 0.02 and 0.05 (Chen et al., 2003; Polli et al., 2003).

The $K_{p,u}$ was $0.44 \pm 0.09$ and $0.29 \pm 0.07$ for S- and R-CZE, respectively. Though the difference indicated by the $K_p$ ratio was reduced, the $K_{p,u}$ values of the two enantiomers were still significantly different ($p=0.0005$). Since $K_{p,u}$ accounts for the differences in binding to blood components (Equation 4) these values indicates that the stereoselectivity in brain partitioning observed with $K_p$ is mainly explained by the difference in plasma protein binding. However, factors other than plasma protein binding are likely to be involved. Transport across the BBB and/or binding in the brain could also be stereoselective. The $K_{p,u}$ for racemic CZE has previously been reported to be 0.23 measured up to 4 h in mice (Polli et al., 2003).

![Figure 9](image-url)

**Figures 9.** The three partition coefficient $K_p$, $K_{p,u}$, $K_{p,uu}$ of S- and R-cetirizine (average ± SD, n = 8) measuring brain distribution of the enantiomers in guinea pigs. Both $K_p$ and $K_{p,u}$ for S-cetirizine was significantly higher than for R-cetirizine ($***p < 0.01$).

Since $K_{p,uu}$ is calculated from the unbound concentrations on both sides of the BBB (Equation 5) it more closely describes the transport across the BBB independent of binding in the blood and brain (Hammarlund-Udenaes et al., 1997), and can therefore directly measure any stereoselectivity in active transport across the BBB. The $K_{p,uu}$ value of $0.17 \pm 0.06$ and $0.14 \pm 0.04$, were not statistically significant between S- and R-CZE, respectively. A $K_{p,uu} < 1$ indicates that active efflux processes are acting on CZE at the BBB (Hammarlund-Udenaes et al., 1997). These results are consistent with the findings of Chen et al. and Polli et al., who showed that P-gp is involved in the efflux of CZE at the BBB (Chen et al., 2003; Polli et al., 2003).
The similar K_{p,uu} for S- and R-CZE indicates that the transport across the BBB is the same and that the active efflux at the BBB does not differ between the two enantiomers. Our results fit with the results from Whomsley et al., who showed no difference in the transport characteristics of racemic CZE and R-CZE in Caco-2 cell monolayers. S-CZE was not studied separately (Whomsley et al., 2003).

The binding of CZE enantiomers within the brain was measured with V_{u,br}. It compares the total brain concentration of a drug with its unbound ISF concentration and therefore describes the distribution within the brain irrespective of blood to brain distribution. The V_{u,br} of 2.86 ± 0.88 ml/g brain for S-CZE was not significantly different from 2.39 ± 0.79 ml/g brain for R-CZE (Figure 10) indicating no difference in the intracellular distribution and/or binding of CZE enantiomers within the brain. A significant difference observed for the K_{p,u} could be explained by Equation 4. The K_{p,u} is a product of V_{u,br} (1/f_{u,brISF}) and K_{p,uu}. The V_{u,br} and K_{p,uu} are not different for the two enantiomers, however there is trend of both being higher for S-CZE leading to a significant difference in the K_{p,u} value. If the brain ISF volume of guinea pigs is assumed to be similar to that observed in other animal species (Levin et al., 1970; Goodman et al., 1973), a V_{u,br} of approximately 2.5 ml/g brain indicates that the CZE enantiomers distribute intracellularly in the brain and/or bind readily to tissue components in the extracellular space.

**Figures 10.** Intra-brain distribution measured by unbound volume of distribution, V_{u,br} of S- and R-cetirizine (average ± SD, n = 8).

The terminal half-life of CZE enantiomers in the brain, based on unbound concentrations was significantly longer than in the plasma (p=0.008; Table 3). In Paper IV, the terminal half-life of R-CZE in the brain (5.64 h and 4.03 h for the 0.1 and 1 mg/kg doses, respectively) and plasma (2.83 and 2.15 h, respectively), based on total concentrations were similar to the values obtained in Paper III.
Concentration vs. H₁ Receptor Occupancy of Levocetirizine (Paper IV)

For antihistamines H₁ receptor occupancy in the brain is an indicator of their central side effects and is commonly measured at the time of maximum plasma concentration (Wiech and Martin, 1982; Snowman and Snyder, 1990; Kreutner et al., 2000; Yakuo et al., 2001). For levocetirizine, the pharmacokinetics in brain differs from plasma pharmacokinetics and this prompted us to investigate and compare the time course of H₁ receptor occupancy in the periphery to that in brain.

To carry out H₁ receptor occupancy studies in the brain and peripheral tissues with the drug ex-vivo, we have taken advantage of the fact that levocetirizine has a long residence time on the H₁ receptor due to very slow dissociation kinetics (dissociation half-life =142 min, Gillard et al. 2002) In this respect we studied central H₁ receptor occupancy using displacement of [³H]-mepyramine binding to the cerebellum and peripheral occupancy using the shift of histamine concentration-response curves in segments of ileum obtained from the same guinea pigs. However, it should be noted that this shift could be underestimated due to the time elapsed between the sacrifice of the animal and the *in vitro* experiment which included a 20 min stabilization period in the organ bath.

![Figure 11. The H₁ receptor occupancy versus time profiles in ileum and brain following oral administration of 0.1 and 1 mg/kg levocetirizine in guinea pigs. Each point represents Mean ± SD of four animals.](image)

One hour after administration, 0.1 mg/kg dose of levocetirizine induced a H₁ receptor occupancy of 75 % in the periphery (Figure 11). Under the same conditions, this occupancy was less than 20 % at all time points in the brain. An occupation of approximately 30 % of histamine H₁ receptors in the human cerebral cortex (Yanai et al., 1999) seems to be tolerated before the emergence of the side effects. This demonstrates that levocetirizine has an effective antihistamine effect without central adverse effects. At the dose of 1 mg/kg, which is 10 times the therapeutic dose, the H₁ receptor in the periphery remained highly occupied up to 8 h post dosing (90-96 %). In the brain, the H₁ receptor occupancy was maximal at 2 h
Figure 12. The plasma concentrations vs. ileum H₁ receptor occupancy curve of levocetirizine after oral administration of 0.1 mg/kg and 1 mg/kg in guinea pigs.

Figure 13. a) Plasma concentrations vs. brain H₁ receptor occupancy curve b) Brain concentrations vs. brain H₁ receptor occupancy curve of levocetirizine, after the oral administration of 0.1 mg/kg and 1 mg/kg doses in guinea pigs.
after dosing and remained at the same level until 8 h. It ranged from 30 to 75 %
during the 16 h study period. In rats, the H<sub>1</sub> receptor occupancy by racemic CZE
has been reported to be 22.5 and 34.2 %, one hour after doses of 10 and 30 mg/kg
i.p., respectively (Snowman and Snyder, 1990). Toshiro et al. measured H<sub>1</sub> receptor
occupancy in eight healthy volunteers by PET using [<sup>11</sup>C]-doxepin 90 min after a
relatively high 20 mg oral CZE dose (the recommended daily dose of cetirizine is
10 mg orally) and showed that cetirizine occupied 30 % of the H<sub>1</sub> receptors (Tashiro
et al., 2002).

The plasma concentration vs. ileum H<sub>1</sub> receptor occupancy curve showed no
delay in concentration-effect relationship in the periphery (Figure 12). A plot of the
plasma concentration vs. H<sub>1</sub> receptor occupancy in the brain, however, indicated that
the central H<sub>1</sub> receptor occupancy lag behind the plasma concentration resulting in
counterclockwise hysteresis (Figure 13a). Plotting the H<sub>1</sub> receptor occupancy in brain
against brain concentration resulted in a collapse of the hysteresis loop (Figure 13b).
This indicates that the delay in the brain H<sub>1</sub> receptor occupancy curve compared to
plasma concentrations is solely caused by a slow transport across the BBB.
In conclusion, the pharmacokinetics of CZE enantiomers was stereoselective in the guinea pig. Differences in the protein binding of the two enantiomers was the primary factor affecting the pharmacokinetics. The effect of protein binding as an important factor influencing the differences in pharmacokinetics of the CZE enantiomers could be extrapolated to humans.

A conclusion regarding stereoselectivity in brain distribution has several components, BBB equilibration, binding in brain tissue and blood and is dependent on the chosen parameter. While the differences in $K_p$ and $K_{p,u}$ between R- and S-CZE could be interpreted as a stereoselective brain distribution, this is not the case when the determining factors are separated. There is no difference in $K_{puu}$, i.e. the BBB equilibrium between the enantiomers, showing that there is no enantiomeric differences in active transport across the BBB. There is neither any difference in brain tissue distribution and binding, although both the enantiomers are extensively distributed intracellularly and/or bound to ISF protein in brain. The difference observed in $K_p$ is only caused by difference in plasma protein binding. Thus, when determining stereoselectivity in brain distribution it is important to study all factors governing this distribution.

For levocetirizine, the delay in $H_1$ receptor occupancy could be fully explained by the delay in distribution caused by slow BBB transport. In guinea pigs, a dose of levocetirizine (0.1 mg/kg) producing effective blockade of histamine $H_1$ receptors in the periphery had no effect on such receptors in the brain. Higher doses of levocetirizine (1 mg/kg) were necessary to produce an effect at central sites. The present results demonstrate that it is important to characterize the time course of central effect in relation to the brain concentrations of drugs. Furthermore, preclinical investigation of brain $H_1$ receptor occupancy at the time of maximum plasma concentration is not sufficient especially when the pharmacokinetics of a drug in the brain is different from that in the plasma.
Anubha Gupta

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References


Beal SL and Sheiner LS (1994) NONMEM user’s guide, NONMEM Project Group, University of California at San Francisco, San Francisco.


BBB and cetirizine enantiomers


Anubha Gupta


BBB and cetirizine enantiomers


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