The distribution of the HIV protease inhibitor, ritonavir, to the brain, cerebrospinal fluid and choroid plexuses of the guinea-pig[†]

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Non standard abbreviations: 3TC, (-)-β-L-2',3' dideoxy-3'thiacytidine; AZT, 3'-azido 3'-deoxythymidine; CSF, cerebrospinal fluid; d4T, 2',3' didehydro-3'-deoxythymidine; ddC, 2',3'-dideoxycytidine; DMSO, dimethyl sulfoxide; dpm, disintegrations per minute; HIV, human immunodeficiency virus; K_{in}, unidirectional transfer constant; MRP, multidrug resistance associated protein; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; OAT, organic anion transporter; Oatp, organic anion transporting polypeptide; P-gp, P-glycoprotein.

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Abstract

Anti-HIV drug penetration into the brain and cerebrospinal fluid (CSF) is necessary to tackle HIV within the CNS. This study examines movement of [^{3}H]ritonavir across the guinea-pig blood-brain and blood-CSF barriers and accumulation within the brain, CSF and choroid plexus. Ritonavir is a protease inhibitor, used in combination therapy (often as a pharmacoenhancer) to treat HIV. Drug interactions at brain barrier efflux systems may influence the CNS penetration of anti-viral drugs, thus the influence of additional protease inhibitors, nucleoside reverse transcriptase inhibitors and non-nucleoside reverse transcriptase inhibitors on [^{3}H]ritonavir CNS distribution was explored. Additionally, the involvement of transporters on [^{3}H]ritonavir passage across the brain barriers was assessed. Results from *in situ* brain perfusions and capillary depletion analysis demonstrated that [^{3}H]ritonavir uptake into the guinea-pig brain was considerable (6.6±0.7ml/100g at 30 min, vascular space corrected), although a proportion of drug remained trapped in the cerebral capillaries and did not reach the brain parenchyma. CSF uptake was more limited (2.2±0.4ml/100g at 30 min), but choroid plexus uptake was abundant (176.7±46.3ml/100g at 30 min). [^{3}H]ritonavir brain and CSF uptake was unaffected by inhibitors of organic anion transport (probenecid and digoxin) or P-glycoprotein (progesterone), nor by any additional anti-HIV drugs, indicating that brain barrier efflux systems do not significantly limit brain or CSF [^{3}H]ritonavir accumulation in this model. [^{3}H]ritonavir uptake into the perfused choroid plexus was significantly reduced by nevirapine and abacavir, additional perfusion studies and isolated incubated choroid plexus experiments were carried out in an attempt to further characterise the transporter involved.

Ritonavir is a human immunodeficiency virus (HIV) protease inhibitor which exhibits potent activity against HIV-1 and HIV-2 (Kempf et al., 1995). Conventional doses of ritonavir (600 mg, twice daily) are not very well tolerated, side effects include nausea, diarrhoea, taste alterations and perioral paraethesia (Moyle and Back, 2001), and high rates of discontinuation are reported (Rublein et al., 1999). However, lower ritonavir doses (100-200 mg) are better tolerated, and the use of low dose ritonavir as a pharmacoenhancer of other protease inhibitors, is becoming widespread (Rathbun and Rossi, 2002). Pharmacoenhancement improves the plasma pharmacokinetics of the boosted drug, giving rise to plasma levels above the 50% inhibitory concentration value. This increases the antiviral effects of the drug, reduces the likelihood of drug-resistant strains of virus developing, and allows less frequent dosing (Moyle and Back, 2001; Rathbun and Rossi, 2002). The boost is achieved by inhibition of metabolising enzymes, and possibly efflux transporters, in the intestines and liver, which increases the bioavailability of the boosted drug. Ritonavir has a very high affinity for the CYP3A and CYP2D families of metabolising enzymes, which are highly expressed in the liver and intestines (Kumar et al., 1996), and can inhibit the metabolism of other anti-HIV protease inhibitors to increase their bioavailability (Kempf et al., 1997; Huisman et al., 2001).

The practice of pharmacoenhancement is additionally thought to exert effects on the CNS penetration of anti-viral drugs. The effective penetration of anti-HIV drugs into the brain and cerebrospinal fluid (CSF) is required to tackle HIV within the CNS. Without therapeutic drug levels at this site, HIV replication can continue and the CNS can become a viral reservoir (Schrager and D'Souza, 1998). Furthermore, viral replication in the presence of sub-therapeutic drug levels can lead to the emergence of

drug-resistant viral strains, which may leave the CNS and enter the periphery, leading to therapy failure (Cunningham et al., 2000). Ritonavir distribution into human CSF is limited (less than 50 ng/ml) (Hsu et al., 1998). However, it is well established that due to structural and functional differences between the blood-brain barrier (found at the level of the cerebral capillary endothelial cells) and the blood-CSF barrier (the choroid plexuses and arachnoid membrane), drug levels in the CSF do not necessarily reflect levels in the brain (Thomas and Segal, 1998; Thomas et al., 2001; Gibbs and Thomas, 2002). Transport of ritonavir across the *in vitro* blood-brain barrier has been explored using an astocyte/endothelial cell co-culture model (van der Sandt et al., 2001), and it was determined that ritonavir exhibited polarised transport across this barrier. However, ritonavir distribution into the brain remains to be investigated. Using the guinea-pig *in situ* brain perfusion and isolated incubated choroid plexus methods, this study examines the ability of [^{3}H]ritonavir to cross the brain barriers and accumulate within the brain, CSF and choroid plexus.

Pharmacoenhancement may influence the CNS penetration of anti-viral drugs as a result of drug interactions at brain barrier efflux transporters. Transporters are expressed at the choroid plexuses and the blood-brain barrier (Ghersi-Egea and Strazielle, 2002; Kusuhara and Sugiyama, 2001) which limit the penetration of many drugs, including anti-HIV therapies, into the CNS (Gibbs and Thomas, 2002; Gibbs et al., 2003a; Gibbs et al. 2003b). A great body of evidence suggests that ritonavir is a substrate for efflux transporters. The majority of these studies have focused on the interaction between ritonavir and two families of ATP-binding cassette transporters: P-glycoprotein (P-gp) and multidrug resistance associated proteins (MRPs), both are present at the brain barriers. Ritonavir is reported to be an inhibitor (if not a

substrate) for P-gp, MRP1 and MRP2 (Lee et al., 1998; Jones et al., 2001; Huisman et al., 2002). Furthermore, interactions are reported to occur between ritonavir and other protease inhibitors at the site of brain barrier efflux transporters (van Praag et al., 2000; Drewe et al., 1999) resulting in altered drug concentrations within the CNS. Here, we build on this theme to explore the influence of nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors on the CNS distribution of [^{3}H]ritonavir, and the involvement of transporters in [^{3}H]ritonavir movement across the brain barriers. An advantage of using the brain perfusion technique is that known levels of [^{3}H]ritonavir can be perfused into the CNS, in the absence or presence of transport inhibitors or unlabelled anti-HIV drugs, without the interference of systemic metabolising enzymes which may function to alter plasma drug levels, allowing direct determination of the influence of brain barrier processes on [^{3}H]ritonavir CNS accumulation.

Methods

In situ brain perfusions

In situ brain perfusions were carried out on anaesthetised (intramuscular: 0.32 mg/kg fentanyl, 10 mg/kg fluanosine and 5 mg/kg midazolam) and heparinised (25 000 units/ml) adult Dunkin-Hartley guinea-pigs (250-300 g). As described previously (Gibbs and Thomas, 2002), the left and right carotid arteries of the guinea-pig were perfused with an artificial plasma containing radiolabelled ritonavir ([^{3}H]ritonavir: 115 nM) and sucrose ([^{14}C]sucrose: 160 nM) for up to 30 min. At the end of the perfusion, the CSF was sampled from the cisterna magna before the animal was decapitated and the brain removed. The cerebrum was sampled and the left and right choroid plexuses extracted. These samples, along with the CSF sample and aliquots of the artificial plasma were solubulised (Solvable; Packard, Berkshire, UK) and the radioactivity in the samples measured using liquid scintillation counting. [^{3}H]ritonavir and [^{14}C]sucrose uptake into the CNS was determined by calculating the radioactivity (disintegrations per minute; dpm) in the CNS samples (dpm/g) as a percentage of radioactivity in the artificial plasma (dpm/ml) and is expressed as uptake % (ml/100g).

Multiple-time uptake studies

In multiple-time uptake experiments, artificial plasma containing [^{3}H]ritonavir and [^{14}C]sucrose was perfused into the cannulated left and right carotid arteries of the guinea-pigs for 2.5, 10, 15, 20 or 30 min.

Capillary depletion analysis

Capillary depletion analysis (Triguero et al., 1990) was carried out to establish the extent of [^{3}H]ritonavir uptake into the brain parenchyma. As described previously (Gibbs and Thomas, 2002), brain tissue (0.5 g) was homogenised with 1.5 ml physiological buffer, before addition of 2 ml 26% dextran solution and further homogenisation. The homogenate was then centrifuged (15 min, 4 ºC, 5400 g) to separate the sample into a supernatant (containing the brain parenchyma) and a capillary rich pellet. The radioactivity in the homogenate, supernatant and pellet was determined by liquid scintillation counting.

Self-inhibition studies

Self-inhibition studies were undertaken to examine the effects of excess ritonavir (10 μM) on [^{3}H]ritonavir uptake, and to establish whether a saturable transporter facilitated uptake into or removal from the CNS. For these experiments, a 1 mM ritonavir stock solution was made up in ethanol, which was then diluted with the artificial plasma to produce a final concentration of 10 μM (final ethanol level 1%). This artificial plasma was perfused simultaneously with [^{3}H]ritonavir and [^{14}C]sucrose for 10 min. Results from these perfusions were compared with results from control experiments where an artificial plasma containing 1% ethanol was perfused into the carotid arteries for 10 min.

Cross competition studies

To evaluate the effects of additional drugs on the CNS uptake of [^{3}H]ritonavir, NRTIs, NNRTIs and protease inhibitors were co-perfused with [^{3}H]ritonavir and [^{14}C]sucrose. Brain perfusion studies with NRTIs were carried out over 20 min in the

presence of: abacavir, 3'-azido 3'-deoxythymidine (AZT), 2',3'-dideoxycytidine (ddC), 2',3' didehydro-3'-deoxythymidine (d4T) or (-)-β-L-2',3' dideoxy-3'thiacytidine (3TC). Abacavir, AZT, ddC and d4T were dissolved into the artificial plasma to achieve a final concentration of 100 μM, 3TC was used at 50 μM. Due to the limited availability of the unlabelled NNRTIs (nevirapine and efavirenz) and protease inhibitors (ritonavir and saquinavir), it was necessary to confine the length of cross competition studies with these drugs to 10 min. Nevirapine and efavirenz, were added to the artificial plasma to achieve final concentrations of 100 μM. The limited solubility of these drugs meant that it was necessary to first make 100 mM stock solutions in dimethyl sulfoxide (DMSO), and then dilute these to the required concentration in the artificial plasma. The plasma level of DMSO in these experiments was 0.1%. [^{14}C]sucrose uptake, over 10 min, into the cerebrum, choroid plexus and CSF (0.9 ± 0.02 ml/100g; 10.2 ± 0.9 ml/100g; and 0.2±0.07 ml/100ml, respectively) was not significantly altered by the presence of DMSO, indicating that this solvent did not affect the integrity of the CNS barriers. To assess the effects of saquinavir on [^{3}H]ritonavir CNS accumulation, this protease inhibitor was first dissolved into a 5 mM solution in ethanol, which was then added to the artificial plasma to achieve a final concentration of 50 μM (final ethanol concentration 1%). Results from these studies were compared to control studies in the presence of 1% ethanol.

Transport inhibition studies

Transport inhibitors were added to the artificial plasma, to determine their effects on the CNS uptake of [^{3}H]ritonavir (measured over 20 min). These inhibitors were chosen for their abilities to interact with drug transporters present at the brain barriers,

and the concentrations utilised were based on the published half saturation or half inhibition constants where known. Probenecid (350 μM), digoxin (25 μM) and progesterone (25 μM) were dissolved into the artificial plasma and co-perfused with [^{3}H]ritonavir and [^{14}C]sucrose. Probenecid was first diluted with DMSO (to achieve a 350 mM solution) and progesterone was first diluted with ethanol (to achieve a 50 mM solution), these stock solutions were then added to the artificial plasma to achieve the final concentrations required. In the probenecid and progesterone studies, final DMSO and ethanol levels in the plasma were 0.1% and 0.05%, respectively.

HPLC and radiodetector analyses

HPLC and radiodetector analyses were carried out using a Jasco HPLC system (HG-1580 high pressure, high performance gradient HPLC solvent delivery system, AS-1555-10 cooled autosampler and UV-1575 UV/vis detector; Jasco, Great Dunmow, Essex, UK) and Packard radiodetecter (Radiomatic 525TR analyser; Packard, Pangbourne, UK). The UV absorbance of ritonavir was measured over 20 min at 239 nm using a 150 x 4.6 mm, 3 μm luna C18(2) column (Phenomenex, Cheshire, UK) and an isocratic gradient (52% acetonitrile in water degassed with helium) flowing at 1 ml/min (Penzak et al., 2001). Outflow from the column was directed to the radiodetector where it was mixed with scintillation fluid (Ultima Flo M; Packard) and passed through a 0.5 ml flow cell for real time radioactive analysis.

Ritonavir standards (100 μM) and samples of the inflowing artificial plasma from brain perfusion experiments were prepared for HPLC/radiodetector analyses as follows (Penzak, et al., 2001). 50 μl 0.3 M $BaOH_2$ and 50 μl 0.4 M $ZnSO_4.7H_2O$ were added to the sample (200 μl), which was then vortexed. 800 μl acetonitrile was

added before further vortexing and then centrifugation (15 min, 3000 g, 21 °C). The resultant supernatant was lyophilised to dryness with nitrogen and the sample was then prepared for HPLC/radiodetector analysis by the addition of 200 µl of the mobile phase, vortexing and further centrifugation (10 min, 3000 g, 21 °C). Venous outflow, collected after 25 min of carotid artery perfusion was centrifuged (10 min, 3000 g, 21 °C) and the supernatant removed and prepared for analysis as described above. Samples of brain taken after 30 min [^{3}H]ritonavir perfusions, and also both lateral ventricle choroid plexuses, were prepared for HPLC/radiodetector analysis as described by Thomas and colleagues (Thomas et al., 2001).

Octanol-saline partition coefficient and protein binding

The octanol-saline (pH 7.4) partition coefficient for [^{3}H]ritonavir was determined as described previously (Williams et al., 1996). The extent of [^{3}H]ritonavir binding to proteins in the perfusion medium was determined using ultrafiltration centrifugal analysis (Williams et al., 1996).

Isolated incubated choroid plexus technique

Adult Dunkin-Hartley guinea-pigs (250-300 g) were anaesthetised and then heparinised as described above. The isolated incubated choroid plexus procedure (Gibbs and Thomas, 2002) was then carried out as outlined below. The blood in the choroid plexuses was removed by perfusion of the left ventricle of the heart with physiological saline. At the start of perfusion the right atrium was cut to allow blood outflow. The animal was then decapitated, the brain removed and both lateral ventricle choroid plexuses taken. The choroid plexuses were placed in ice-cold artificial CSF for 20 min, then transferred to warm artificial CSF (37 ºC), in the

absence or presence of unlabelled anti-HIV drugs or transport inhibitors as described previously (ritonavir, saquinavir AZT, abacavir, ddC, 3TC, D4T, nevirapine, probenecid, progesterone and digoxin) as well as lopinavir (100 μM), verapamil (20 μM) or vinblastine (5 μM) for a 10 min incubation period. Stock solutions of verapamil and lopinavir were made in ethanol and DMSO, respectively, and then diluted to the required concentration with CSF, resulting in a final ethanol concentration of 0.1% and a final DMSO concentration of 1%. Subsequently the tissue was transferred to warm artificial CSF (37 ºC), containing [^{3}H]ritonavir (0.96 μM), [^{14}C]sucrose (0.63 μM) and the drugs or inhibitors as described above. After 5 min, the tissue was removed and weighed. The tissue and 50 μl aliquots of artificial CSF, which were taken prior to tissue incubation, were then prepared for radioactive counting.

Measurement of radioactivity and expression of results

Samples were prepared for scintillation counting as described before. Accumulation of [^{3}H]ritonavir, corrected for [^{14}C]sucrose, into the choroid plexus was expressed as a tissue-to-medium ratio (Gibbs and Thomas, 2002).

Materials

[^{3}H]ritonavir (MW 721; specific activity 1.6 Ci/mmol) and D-[^{14}C]sucrose (MW 342 specific activity 0.62 Ci/mmol) were purchased from Moravek Biochemicals Inc. (California, USA) and Amersham Pharmacia Biotech UK Ltd (Buckinghamshire, UK), respectively. Unlabelled anti-HIV drugs were purchased from Moravek Biochemicals Inc. or Sigma Biochemicals (Dorset, UK), except nevirapine and efavirenz which were kindly provided by Boehringer-Ingelheim Pharmaceuticals, Inc.

(Connecticut, USA) and Bristol-Myers Squibb Company (Princeton, USA), respectively. Unless specified all other chemicals were from Merck Ltd (Lutterworth, UK).

Data analysis

The data from all the experiments are presented as mean±SEM. Following multiple-time uptake studies, the slopes (K_{in}) of the lines were determined by least squares regression analysis, where appropriate, and are reported together with the correlation coefficient (r) and the level of significance that time can be used to predict the uptake value. Statistical analysis was performed using SigmaStat 2.0 software (SPSS Science Software UK Ltd, Birmingham, UK) and significance was taken as follows: not significant (ns) $p>0.05$, * $p<0.05$ and ** $p<0.01$.

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Results

Multiple-time uptake studies

Uptake of [^{3}H]ritonavir and [^{14}C]sucrose into the brain, CSF and choroid plexus over 2.5-30 min is shown in Figure 1. [^{3}H]ritonavir uptake into the brain was considerable, reaching 8.7 ± 0.8 ml/100g by 30 min, and could be described by a linear distribution, with a K_{in} value of 3.2 ± 0.4 μl/min/g ($p<0.001$, $r=0.827$). [^{14}C]sucrose brain uptake could also be described by linear regression with a K_{in} value of 0.7 ± 0.09 μl/min/g ($p<0.005$, $r=0.785$). The rate of [^{14}C]sucrose entry into the brain was significantly lower than the rate of [^{3}H]ritonavir entry (2 sample t test, $p<0.001$)

[^{3}H]ritonavir uptake into the CSF was markedly lower than brain uptake, reaching 2.2 ± 0.4 ml/100g after 30 min. [^{14}C]sucrose CSF uptake at 30 min was 1.9 ± 0.5 ml/100g, and was not significantly different to [^{3}H]ritonavir uptake (paired t test, $p>0.05$). In fact, uptake of [^{3}H]ritonavir into the CSF did not exceed uptake of the marker molecule at any time point (paired t tests, $p>0.05$).

In comparison to the limited CSF uptake of [^{3}H]ritonavir, choroid plexus uptake was abundant, reaching 176.7 ± 46.3 ml/100g after 30 min. At each time point, drug accumulation in the tissue was significantly greater than accumulation of the extracellular space marker [^{14}C]sucrose (paired t-test and Wilcoxon signed rank test, $p<0.05$).

HPLC and radiodetector analyses

[^{3}H]ritonavir extraction from the artificial plasma before (arterial inflow) and after (venous outflow) perfusion through the guinea-pig carotid arteries is shown in Figure 2. These results confirmed that intact [^{3}H]ritonavir was presented to the brain, and remained intact throughout the passage through the cerebral circulation. Additionally, intact [^{3}H]ritonavir could be detected in brain and choroid plexus tissue, sampled after a 30 min perfusion.

Capillary depletion analysis

Results from capillary depletion analysis performed after 30 min brain perfusions are shown on Figure 3. The extent of [^{3}H]ritonavir uptake (corrected for [^{14}C]sucrose) into the whole brain (homogenate), brain parenchyma (supernatant) and cerebral capillary rich pellet was comparable (one way ANOVA, $p>0.05$). Results clearly demonstrated that [^{3}H]ritonavir can cross the blood-brain barrier endothelial cells and accumulate within the brain parenchyma ([^{3}H]ritonavir uptake into the supernatant was 4.5 ± 1.3 ml/100g). However, the substantial accumulation of [^{3}H]ritonavir in the cerebral capillary rich pellet (6.5 ± 2.0 ml/100g) indicates that a proportion of the drug remains trapped in the capillaries and does not penetrate any further into the cerebrum.

Cross competition studies

As part of the cross competition studies, unlabelled ritonavir and saquinavir were dissolved in ethanol before use, resulting in a final plasma concentration of 1% ethanol. Figure 4 shows the effects of perfusion with an artificial plasma containing 1% ethanol on [^{3}H]ritonavir and [^{14}C]sucrose CNS uptake. Ethanol did not affect the

permeability of the brain barriers to [^{14}C]sucrose (t tests, $p>0.05$). However, a significant reduction in [^{3}H]ritonavir uptake into the choroid plexus was noted when ethanol was added to the perfusion medium (t test, $p<0.01$). Consequently, the effects of unlabelled ritonavir and saquinavir on [^{3}H]ritonavir CNS uptake over 10 min were compared back to the 10 min perfusions in the presence of ethanol (Table 1).

Table 1 shows the effects of the NRTIs, NNRTIs and protease inhibitors on [^{3}H]ritonavir CNS accumulation (corrected for [^{14}C]sucrose). [^{3}H]ritonavir uptake into the brain was unchanged in the presence of any of the additional anti-HIV drugs. Abacavir and nevirapine significantly reduced uptake of [^{3}H]ritonavir into the choroid plexus, but none of the drugs significantly affected [^{3}H]ritonavir levels in the CSF.

Transport inhibition studies

Further studies were carried out to determine whether [^{3}H]ritonavir uptake into the brain and choroid plexus would be altered in the presence of transport inhibitors. Table 2 shows the effects of probenecid, progesterone and digoxin on [^{3}H]ritonavir uptake into these regions. Drug uptake into both brain and choroid plexus was unaffected by the inhibitors (One Way ANOVAs followed by Dunnett's method where appropriate, $p>0.05$). However, [^{3}H]ritonavir levels in the brain were reduced from 5.6 ± 0.6 ml/100g to 3.2 ± 0.6 ml/100g in the presence of progesterone, but this attenuation did not attain statistical significance.

Isolated incubated choroid plexus technique

[^{3}H]ritonavir uptake into the isolated choroid plexus ([^{14}C]sucrose/extracellular space corrected) reached 4.9 ± 1.0 ml/g following a 5 min incubation. The effects of additional drugs on [^{3}H]ritonavir and [^{14}C]sucrose uptake are shown on Figure 5. The integrity of the choroid plexus cells throughout the incubated choroid plexus studies was determined by comparing uptake of the extracellular space marker in each experimental group against the control group. No significant differences were seen in [^{14}C]sucrose uptake in the presence of additional anti-HIV drugs (Kruskal-Wallis one-way ANOVA on Ranks or one-way ANOVA with Dunnett's method, as appropriate; Figure 5). Within the group of specific inhibitors, [^{14}C]sucrose uptake in the presence of probenecid was significantly increased from 0.4 ± 0.1 ml/g to 1.5 ± 0.5 ml/g (One way ANOVA followed by Dunnett's method, $p<0.05$; Figure 6).

Uptake of [^{3}H]ritonavir (corrected for extracellular space) was not significantly altered by the presence of any of the NRTIs or protease inhibitors when compared against the control group (one-way ANOVA and Kruskal-Wallis one-way ANOVA on Ranks as appropriate). However, in the presence of 100 μM nevirapine, choroid plexus accumulation of [^{3}H]ritonavir (corrected for [^{14}C]sucrose) was significantly reduced from 4.9 ± 1.0 ml/g to 1.3 ± 0.4 ml/g (t test, $p<0.05$). Figure 6 shows the choroid plexus uptake of radiolabelled ritonavir and sucrose in the absence and presence of the range of transport inhibitors. [^{3}H]ritonavir uptake, corrected for extracellular space, was not significantly altered in the presence of any of these inhibitors.

Octanol-saline partition coefficient and protein binding

The octanol-saline partition coefficient determined for [^{3}H]ritonavir was 22.2 ± 0.7 (n=3). [^{3}H]ritonavir binding to protein in the artificial plasma was 88.3 ± 0.7% (n=3).

Discussion

This study provides novel data relating to the ability of ritonavir to cross the mammalian brain barriers and accumulate within the CNS. Whilst clinical studies have measured ritonavir penetration into CSF, previous studies have not investigated penetration into the brain or choroid plexus. The high level of [^{3}H]ritonavir binding to artificial plasma proteins meant that only 12% of the drug in the plasma was unbound, and free to cross membranes and distribute in anatomical compartments, and this must be considered when interpreting the results. (A high degree of binding to human plasma proteins (99%) has been noted (Boffito et al., 2002)). Multiple-time uptake studies demonstrated that [^{3}H]ritonavir uptake into the guinea-pig brain was considerable, drug levels in the cerebrum approached 9% of plasma levels after 30 min ([^{3}H]ritonavir plasma levels represent both protein bound and unbound drug). The rate of uptake (K_{in}) of [^{3}H]ritonavir into the brain was significantly greater than the rate of uptake of the vascular space marker, [^{14}C]sucrose. Passage of [^{14}C]sucrose across the BBB is limited to paracellular diffusion, and the low lipophilicity of this molecule (octanol-saline partition coefficient: 0.0005 ± 0.00003 (Williams et al., 1996)) greatly limits the rate of this diffusion. In contrast, ritonavir is considerably more lipophilic (octanol-saline partition coefficient: 22.2 ± 0.7) and the significantly faster rate of cerebral uptake may be explained by this characteristic. Self-inhibition studies (Table 1) illustrated that unlabelled ritonavir (10 μM) in the artificial plasma did not affect CNS uptake of [^{3}H]ritonavir, indicating that ritonavir uptake across the BBB is not saturable at this concentration. (Due to the limited solubility of ritonavir, and the other protease inhibitors (Weiss et al., 2002), the achievable concentrations of these drugs in self-inhibition and cross-competition studies was limited.) HPLC and radiodetector analyses (Figure 2) confirmed that intact ritonavir reached the brain.

Although there is evidence to suggest that neurons and astrocytes can be infected with HIV, it is thought that macrophages/microglia are the major cell type in which HIV replicates within the brain (Wiley et al., 1986; Takahashi et al., 1996; Trillo-Pazos et al., 2003). Thus to reduce viral replication within the CNS, anti-HIV agents must be able to target virus within these cells. Capillary depletion analysis measures drug uptake into the brain parenchyma, and thus provides a good indication of whether [^{3}H]ritonavir might reach its target site within the CNS. Data from capillary depletion analysis (Figure 3) illustrated that a portion of the [^{3}H]ritonavir detected in the cerebrum was not in the brain parenchyma, but trapped in the cerebral capillaries. Nevertheless, [^{3}H]ritonavir accumulation in the brain parenchyma was still substantial at 4.5 ± 1.3 ml/100g after 30 min.

The distribution of ritonavir into human CSF is described as low, with concentrations generally less than 50 ng/ml (Hsu et al., 1998). Similarly, ritonavir CSF levels in patients receiving a combination of ritonavir and saquinavir, measured 6-8 h after dosing, ranged from 1.9 to 23 ng/ml (0.1 to 0.5% of plasma levels) (Kravcik et al., 1999). In accord, [^{3}H]ritonavir uptake into the guinea-pig CSF, following 30 min perfusions, was determined to be limited, at 2.2 ± 0.4% of plasma levels, and not significantly higher than uptake of the marker molecule, [^{14}C]sucrose. Given the lipophilicity of [^{3}H]ritonavir, this uptake is lower than expected, and is markedly lower than [^{3}H]ritonavir brain uptake. Although clinical studies are necessarily confined to CSF sampling as an indication of drug levels in the CNS, this study indicates that for ritonavir, such methodology may underestimate drug levels in the brain.

The substantial uptake of [^{3}H]ritonavir into the choroid plexus (Figure 1 and Figure 2) is of interest as the choroid plexus has been indicated as a possible route of entry and viral reservoir for both human and feline immunodeficiency virus (Bragg et al., 2002; Banks et al., 2001). Consequently ritonavir may be a promising drug for the treatment of HIV within the choroid plexus. The fact that [^{3}H]ritonavir accumulation from the blood into the choroid plexus is so substantial, yet CSF drug levels are limited, indicates that [^{3}H]ritonavir movement from choroid plexus to CSF is tightly regulated, and suggests that an efflux process at the choroid plexus may limit drug accumulation in the CSF.

In view of the fact that low dose ritonavir is always co-administered with additional anti-HIV drugs, cross-competition studies were designed to determine the effects of these drugs on [^{3}H]ritonavir CNS accumulation. CSF and brain levels of [^{3}H]ritonavir were unaffected by additional drugs. Of interest, 50 μM saquinavir (a protease inhibitor and P-gp substrate) did not affect [^{3}H]ritonavir brain uptake. Although this opposes findings from Drewe and co-workers, who determined that ritonavir could inhibit P-gp mediated extrusion of saquinavir from cultured brain endothelial cells (Drewe et al., 1999), it is in agreement with work by Huisman and colleagues (2001). Through studies with P-gp knock-out mice and a P-gp expressing epithelial cell line, it was determined that ritonavir (50 mg/kg) did not have a strong inhibitory effect on the activity of blood-brain barrier P-gp towards saquinavir (Huisman et al., 2001). Similarly, it has been established through clinical studies that although administration of ritonavir (100 mg twice daily) increased the CSF penetration of indinavir (a protease inhibitor and P-gp substrate), this increase was due to ritonavir's inhibition of hepatic cytochrome P450 rather than inhibition of brain

barrier P-gp (Haas et al., 2003). So, whilst it has been suggested that P-gp may play an important role in limiting the brain entry of protease inhibitors (Kim et al., 1998; Drewe et al., 1999; van der Sandt et al., 2001), both *in vitro* and *in vivo* studies have indicated that ritonavir is actually a relatively poor inhibitor of blood-brain barrier P-gp, and is not potent enough to sufficiently increase brain concentrations of further protease inhibitors (Polli et al., 1999; Choo et al., 2000; Huisman et al., 2001; van der Sandt et al., 2001; Haas et al., 2003). As discussed earlier, the brain perfusion method can effectively assess the involvement of brain barrier processes on the CNS uptake of drugs, without the interference of systemic metabolising enzymes which may, in other model systems and in the clinical situation, act to alter plasma levels of drug. Hence, these results indicate that neither saquinavir, nor any of the other tested anti-HIV drugs significantly interact with blood-brain barrier processes to alter [^{3}H]ritonavir brain or CSF concentrations. More specifically, this data and results from inhibitor studies with digoxin (a P-gp substrate (Schinkel et al., 1995)) and progesterone (a P-gp modulator (Ueda et al., 1992)) (Table 2), indicates that P-gp does not significantly limit brain accumulation of [^{3}H]ritonavir in this animal model.

During cross competition brain perfusion studies, it was necessary to dissolve ritonavir and saquinavir in ethanol, and results indicated that 1% ethanol affected the permeability of the choroid plexus to [^{3}H]ritonavir (Figure 4). Ethanol has been noted to have an inhibitory effect on CSF production (Javaheri and Corbett, 1998) and it has been postulated that this may be due to ethanol altering choroidal circulation or altering the permeability of choroid plexus tissue. However, it is somewhat surprising that ethanol reduced [^{3}H]ritonavir uptake into the choroid plexus, but not into the brain or CSF. This could be attributed to the fact that [^{3}H]ritonavir enters the choroid

plexus at a very rapid rate, compared to the brain and CSF, and thus the effects of ethanol on barrier permeability are easily seen in this CNS region.

Results from cross competition studies revealed that choroid plexus uptake of [^{3}H]ritonavir, following brain perfusions, was significantly reduced in the presence of abacavir and nevirapine (Table 1). Results from incubated choroid plexus experiments found that nevirapine also reduced the choroid plexus accumulation of [^{3}H]ritonavir from the artificial CSF. This observed reduction would appear to be due to inhibition of a transporter which facilitates [^{3}H]ritonavir tissue accumulation, from either the blood (basolateral membrane) or the CSF (apical membrane). We have previously shown, using the guinea-pig model, that a digoxin-sensitive (Oatp2-like) transport mechanism is involved in the influx of anti-HIV NRTIs from blood to choroid plexus (Gibbs et al., 2003a; Gibbs et al., 2003b). However [^{3}H]ritonavir accumulation in the choroid plexus was insensitive to digoxin (Table 2). A number of efflux transporters are found on the apical membrane of the choroid plexus which facilitate xenobiotic removal from the CSF, and thus into the choroid plexus; these include members of the organic anion transporter (OAT; OAT1 and OAT3) and organic anion transporting polypeptide (Oatp; Oatp1 and Oatp3) families (reviewed in Ghersi-Egea and Strazielle, 2002). The involvement of such a transporter in [^{3}H]ritonavir transport from CSF might explain the limited CSF uptake of this drug. Hence, the possibility that [^{3}H]ritonavir utilises these transporters to accumulate in the choroid plexus was investigated (Figure 6 and Table 2). [^{3}H]ritonavir choroid plexus accumulation was not reduced by any of the inhibitors utilised either *in situ* or *in vitro*. In particular, probenecid (a broad inhibitor of organic anion transporters) did not alter [^{3}H]ritonavir choroid plexus levels, thus the involvement of OAT or Oatp

transporters could not be established. Additionally, vinblastine (a P-gp substrate (Cisternino et al., 2001)), verapamil (a P-gp modulator and MRP inhibitor (van der Sandt et al., 2001)) and progesterone (a P-gp modulator and Oatp2 inhibitor) did not affect [^{3}H]ritonavir choroid plexus accumulation, and it can be concluded that although the literature indicates that ritonavir is a substrate for P-gp, MRP1 and MRP2, these efflux transporters do not seem to influence the choroid plexus uptake of this drug.

In conclusion, this study has shown that [^{3}H]ritonavir can cross the brain barriers to accumulate in the guinea-pig CNS. [^{3}H]ritonavir transport into the brain was not saturable, and is most likely via diffusion. The high lipophilicity of [^{3}H]ritonavir was reflected in the substantial rate of brain and choroid plexus uptake. Uptake of [^{3}H]ritonavir into the CSF was more limited and corresponds to findings in the clinical situation. This study indicates that in the case of ritonavir, using CSF levels as an indicator of CNS penetration may have underestimated levels of drug reaching the brain. Furthermore, this work highlights the importance of the blood-CSF barrier in controlling drug entry into the CNS. [^{3}H]ritonavir levels in the brain and CSF were unaffected by additional anti-HIV drugs. However, in the choroid plexus, abacavir and nevirapine significantly reduced tissue accumulation of [^{3}H]ritonavir, a series of transport inhibition studies could not elucidate any more details about the mechanism behind this interaction.

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Footnotes

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Legends for Figures

Figure 1: *In situ* brain perfusion studies: [^{3}H]ritonavir and [^{14}C]sucrose uptake into the brain, CSF and choroid plexus after 2.5, 10, 15, 20 and 30 min, each time point represents n=3-6.

Figure 2: HPLC/radiodetector analysis of arterial inflow, venous outflow, brain and choroid plexus sampled after a 30min [^{3}H]ritonavir perfusion.

Figure 3: Capillary depletion analysis: [^{3}H]ritonavir uptake (corrected for [^{14}C]sucrose) into the whole brain (homogenate), capillary depleted brain parenchyma (supernatant) and capillary rich pellet after 30 min, n=4. [^{3}H]ritonavir uptake into the homogenate, supernatant and pellet did not significantly differ ($p>0.05$, one way ANOVA).

Figure 4: Uptake of [^{3}H]ritonavir and [^{14}C]sucrose into the guinea-pig CNS following 10 min perfusions with artificial plasma in the absence (control) and presence of 1% ethanol. The effects of ethanol on [^{3}H]ritonavir and [^{14}C]sucrose uptake were assessed using t tests, $^{**}p<0.01$.

Figure 5: Effect of additional anti-HIV drugs on the accumulation of [^{3}H]ritonavir and [^{14}C]sucrose into the isolated choroid plexus, n=3-4. No significant difference was observed between groups after correction for [^{14}C]sucrose space (one way ANOVA and Kruskal-wallis One Way ANOVA, as appropriate)

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Figure 6: Effect of transport inhibitors on [^{3}H]ritonavir and [^{14}C]sucrose accumulation into the isolated incubated choroid plexus, n=3. After correction for [^{14}C]sucrose, no significant difference in [^{3}H]ritonavir accumulation was observed between groups. Probenecid significantly increased the [^{14}C]sucrose/extracellular space ($p<0.05$).

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Table 1: The influence of additional anti-HIV drugs on [^{3}H]ritonavir uptake into the CNS, n=3-5.

Uptake % (ml/100g) [^{14}C]sucrose corrected	*Brain*	*CSF*	*Choroid plexus*
NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS (NRTIs)			
Control (20 mins)	**5.6±0.6**	**0.5±0.2**	**157.1±26.7**
+ *abacavir*	3.9±0.8	0.9±0.3	51.7±18.0 *
+ *AZT*	3.9±0.6	0.5±0.2	117.5±20.0
+ *d4T*	7.7±1.0	Not determined	247.0±81.2
+*ddC*	5.6±1.0	0.1±0.06	270.7±71.6
+*3TC*	4.2±0.6	0.3±0.1	57.9±23.3
NON-NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS (NNRTIs)			
Control (10 mins)	**1.6±0.1**	**0.2±0.04**	**37.8±7.3**
+ *efavirenz*	3.2±0.8	0.6±0.5	46.4±22.7
+ *nevirapine*	1.7±0.6	0.4±0.2	10.6±3.5 *
PROTEASE INHIBITORS			
Control (10 mins, 1% ethanol)	**0.9±0.3**	**0.1±0.05**	**9.7±3.2**
+ *ritonavir*	0.4±0.1	0.1±0.1	7.7±3.4
+ *saquinavir*	0.8±0.2	0.1±0.02	10.6±2.4

The CNS uptake of [^{3}H]ritonavir (corrected for [^{14}C]sucrose) in the presence of NRTIs, NNRTIs and protease inhibitors was compared to the appropriate series of control experiments using one way ANOVA followed by Dunnett's method or Kruskal-Wallis ANOVA on ranks followed by Dunns method, *$p<0.05$.

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Table 2: Uptake of [^{3}H]ritonavir ([^{14}C]sucrose corrected) into the guinea-pig cerebrum and choroid plexus in the absence (control) and presence of transport inhibitors, n=4-6.

	[^{3}H]ritonavir uptake % (ml/100g) ([^{14}C]sucrose corrected)	
	Brain	*Choroid plexus*
Control (20 mins)	**5.6±0.6**	**157.1±26.7**
+ *probenecid*	7.5±1.6	111.0±32.7
+ *progesterone*	3.2±0.6	130.7±42.2
+ *digoxin*	4.2±0.9	266.6±67.4

No changes in drug uptake were seen in the presence of the inhibitors, one way ANOVAs, $p>0.05$.

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Figure 1

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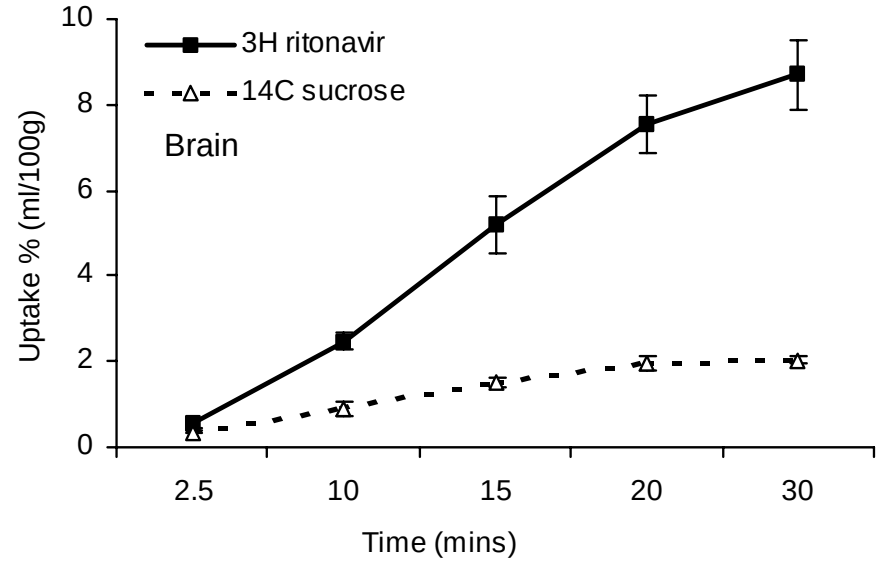

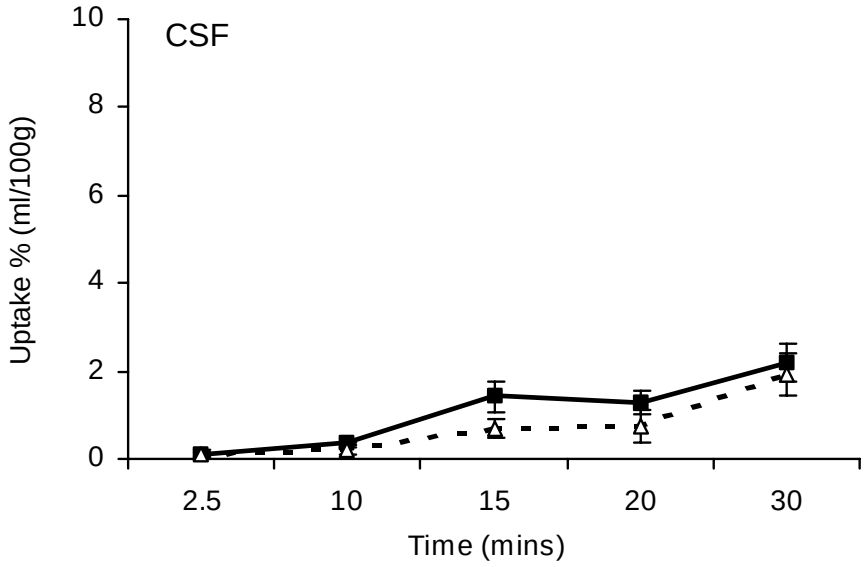

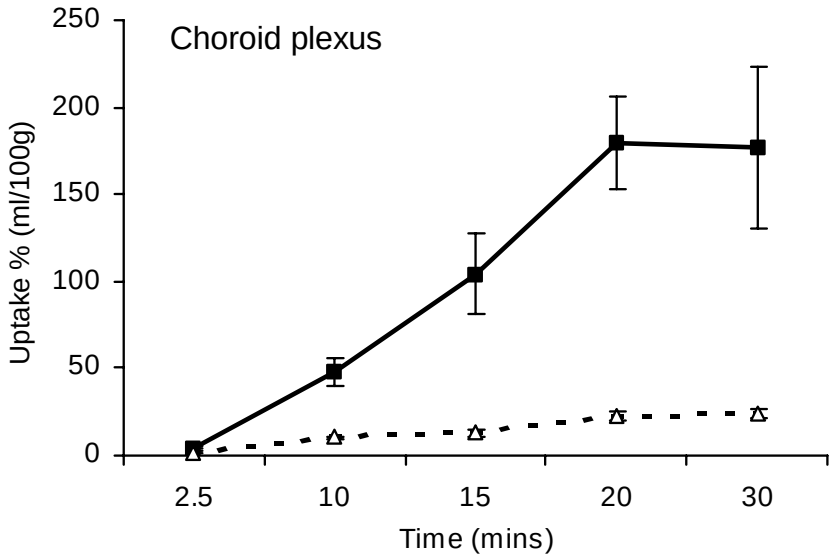

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Figure 2

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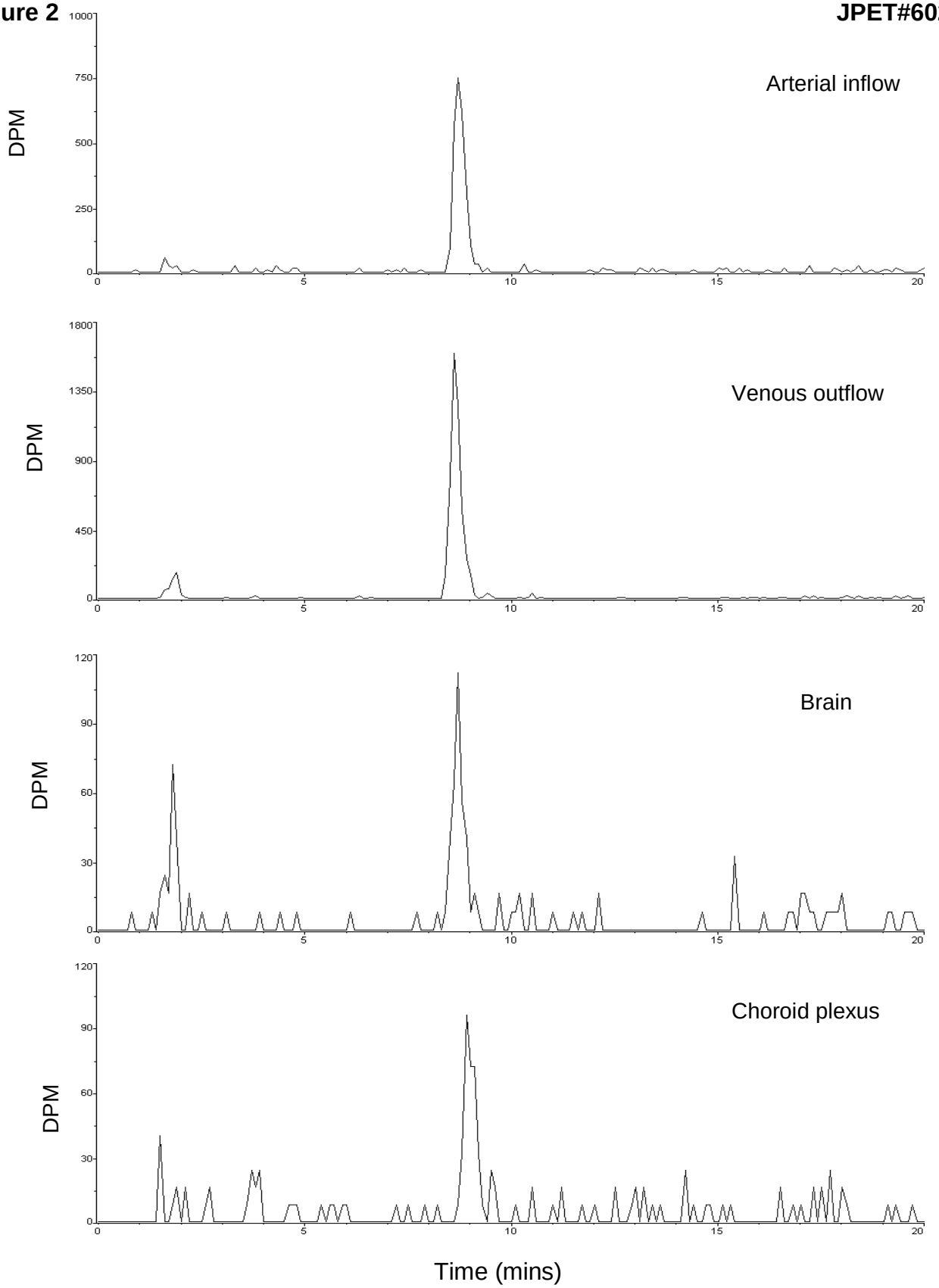

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Figure 3 **JPET#60210**

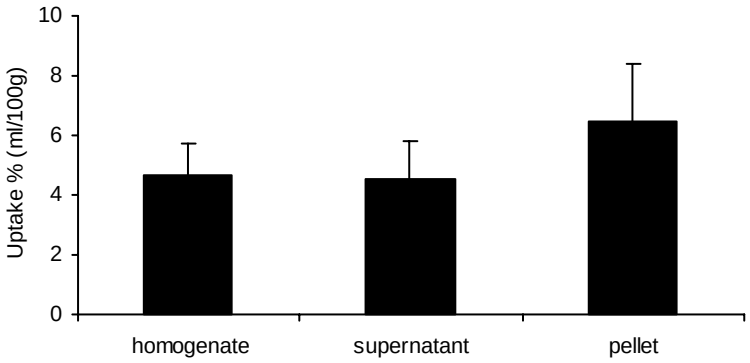

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Figure 4

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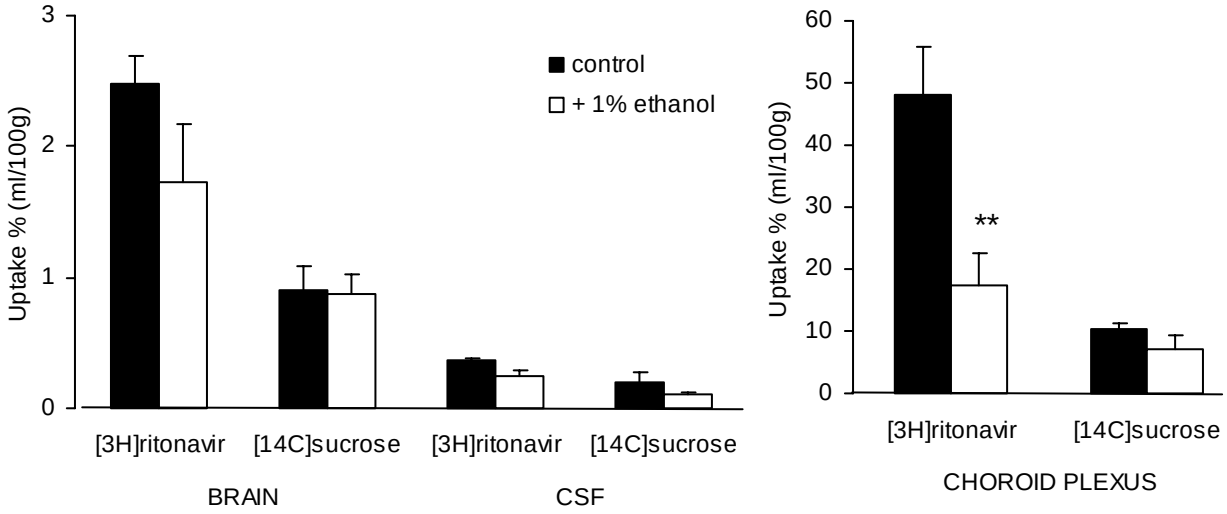

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Figure 5 **JPET#60210**

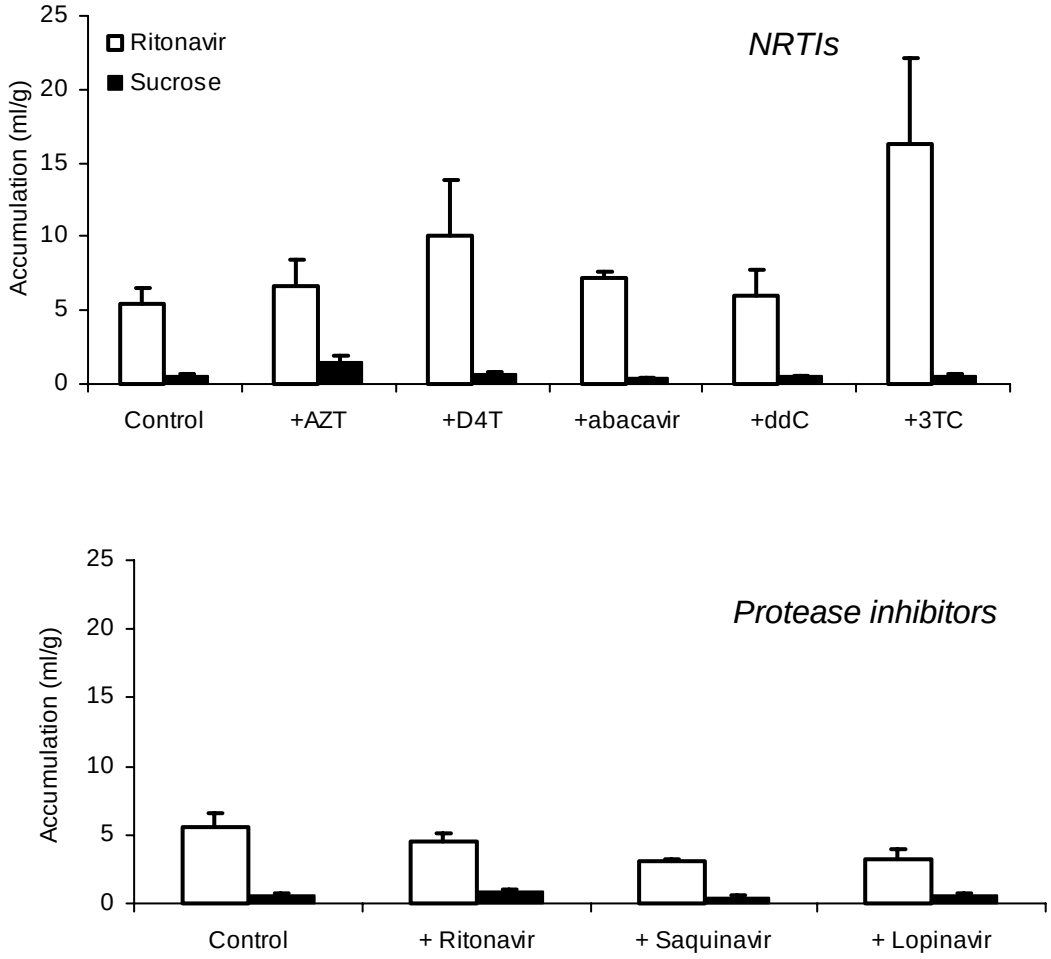

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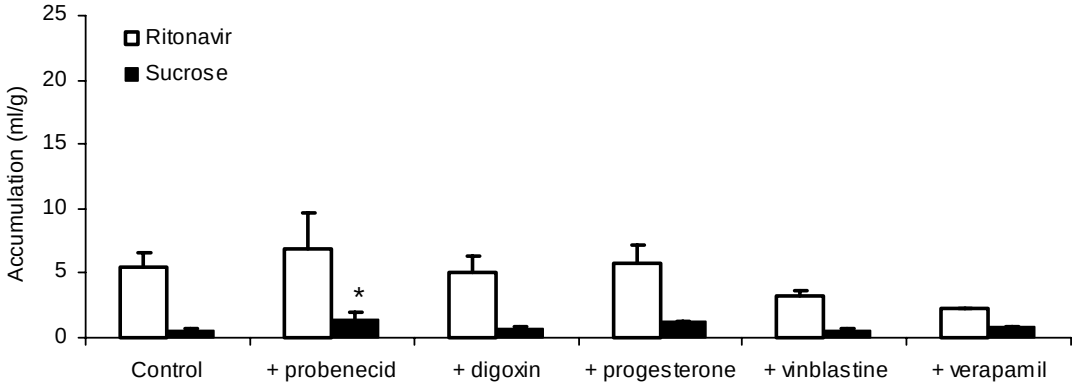
Figure 6 **JPET#60210**



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