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# Modelling of the blood-brain barrier transport of morphine-3-glucuronide studied using microdialysis in the rat: involvement of probenecid-sensitive transport

[1]Rujia Xie, [1]M. René Bouw & *,[1]Margareta Hammarlund-Udenaes

[1]Department of Pharmacy, Division of Biopharmaceutics and Pharmacokinetics, Uppsala University, Box 580, S-751 23, Uppsala, Sweden

**1** The objective of this study was to investigate the impact of probenecid on the blood-brain barrier (BBB) transport of morphine-3-glucuronide (M3G).

**2** Two groups of rats received an exponential infusion of M3G over 4 h to reach a target plasma concentration of 65 $\mu$M on two consecutive days. Probenecid was co-administered in the treatment group on day 2. Microdialysis was used to estimate unbound M3G concentrations in brain extracellular fluid (ECF) and blood. *In vivo* recovery of M3G was calculated with retrodialysis by drug, preceding the drug administration. The BBB transport was modelled using NONMEM.

**3** In the probenecid group, the ratio of the steady-state concentration of unbound M3G in brain ECF to that in blood was $0.08 \pm 0.02$ in the absence and $0.16 \pm 0.05$ in the presence of probenecid ($P = 0.001$). In the control group, no significant difference was found in this ratio between the 2 days ($0.11 \pm 0.05$ and $0.10 \pm 0.02$, respectively). The process that appears to be mainly influenced by probenecid is influx clearance into the brain (0.11 $\mu$l min$^{-1}$ g-brain$^{-1}$ *vs* 0.17 $\mu$l min$^{-1}$ g-brain$^{-1}$, in the absence *vs* presence of probenecid, $P < 0.001$). The efflux clearance was 1.15 $\mu$l min$^{-1}$ g-brain$^{-1}$. The half-life of M3G was $81 \pm 25$ min in brain ECF *vs* $22 \pm 2$ min in blood ($P < 0.0001$). Blood pharmacokinetics was not influenced by probenecid.

**4** In conclusion, a probenecid-sensitive transport system is involved in the transport of M3G across the BBB.

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**Keywords:** Microdialysis; blood-brain barrier; morphine-3-glucuronide; multidrug resistance protein; drug transport

**Abbreviations:** AUC, area under the plasma concentration-time curve; BBB, blood-brain barrier; CSF, cerebrospinal fluid; ECF, extracellular fluid; M3G, morphine-3-glucuronide; MRP, multidrug resistance protein; Pgp, P-glycoprotein; SDS, sodium dodecyl sulphate

## Introduction

Morphine-3-glucuronide (M3G) is the only morphine glucuronide formed in rats and mice (Kuo *et al*., 1991). It is a hydrophilic substance with a log D of $-1.12$ at pH 7.4 (Avdeef, 1996) and only minor penetration across the blood-brain barrier (BBB) has been suggested (Carrupt *et al*., 1991). Total brain-to-plasma concentration ratios of 0.02 (Murphey & Olsen, 1994) and 0.05 (Xie *et al*., 1999) were reported after morphine administration in guinea-pigs and mice, respectively. A cerebrospinal fluid (CSF)-to-plasma ratio of 0.06 has been reported in rabbits (Mignat *et al*., 1995). The BBB permeability surface area product (PS) of M3G is 0.14 $\mu$l min$^{-1}$ g-brain$^{-1}$ in rats (Bickel *et al*., 1996). It has been suggested that the elimination of M3G from brain extracellular fluid (ECF) could take place across the BBB, through the perivascular space into the CSF, and thence into the cervical lymph (Cserr *et al*., 1981). Suggested mechanisms of efflux from the brain include interstitial bulk flow, diffusion and active transport. The precise mechanism that regulated M3G transport across the BBB has not yet been defined. A previous study from this laboratory indicated that the brain ECF-to-blood ratio of M3G does not differ between *mdr1a*($-/-$) and ($+/+$) mice (Xie *et al*., 1999), indicating that P-glycoprotein (Pgp) is not involved. It has also been reported that the BBB transport of M3G was not changed in the presence of the Pgp blocker GF120918 after morphine i.v. bolus administration (Letrent *et al*., 1999). However, since the brain-to-blood ratio is well below unity, it is likely that other active transport systems are involved. At equilibrium, equal concentrations of unbound drug should eventually be achieved on both sides of the BBB, independent of the lipophilicity of the drug, if passive transport is the only mechanism involved (Hammarlund-Udenaes *et al*., 1997 and others). The lower concentrations in brain ECF than in blood could also be ascribed to bulk flow of the brain ECF, metabolism of the drug in the brain, and/or active transport of the drug. The contribution of bulk flow of brain ECF to the difference in concentrations between brain and blood will be greater for drugs with lower rates of passive transport into the brain, i.e. for hydrophilic drugs.

It is well recognized that organic anion transport systems are located at both the choroidal epithelium and the brain capillary endothelium (Pardridge & Oldendorf, 1977; Bradbury, 1979). Many organic anions are pumped out of the brain ECF and CSF by an organic acid pump, which is probenecid-sensitive. Therefore, probenecid can be used to increase the brain concentrations of these drugs. It has been reported that a probenecid-sensitive transport system is involved in the BBB or blood-CSF transport of zidovudine (Wong *et al*., 1993), valproic acid (Adkison *et al*., 1994), baclofen (Deguchi *et al*., 1995), fluorescein (Huai-Yun *et al*., 1998), quinolinic acid (Morrison *et al*., 1999), 17$\beta$-estradol 17$\beta$-D-glucuronide (Nishino *et al*., 1999), and 1-naphthyl-$\beta$-

*Author for correspondence;
E-mail: Margareta.Hammarlund-Udenaes@biof.uu.se

D-glucuronide (Strazielle & Ghersi-Egea, 1999). Since glucuronides are organic anions, the hypothesis of probenecid-sensitive organic anion transport was investigated for the BBB transport of M3G.

The purposes of this study were to investigate the BBB distribution characteristics of M3G and the influence of probenecid on the transport of M3G across the BBB in the rat. A distributional model for the BBB transport of M3G was constructed to describe the data.

## Methods

### *Animals*

Male Sprague-Dawley rats (Charles River, Sweden), weighing 280–320 g, were group housed at 22°C under a 12 h light–dark cycle for at least 1 week before the experiment. Food and water were available *ad libitum*. Ethical approval was obtained from the Animal Ethics Committee of Uppsala University.

### *Chemicals*

Morphine-3-glucuronide was supplied by Lipomed (Arlesheim, Switzerland). Probenecid and low molecular weight heparin were obtained from Sigma (St. Louis, U.S.A.). Enfluran was purchased from the Hospital Pharmacy (Uppsala, Sweden). The perfusion solution (artificial brain ECF) consisted of (mM) NaCl 145, KCl 0.6, $MgCl_2$ 1.0, $CaCl_2$ 1.2 and ascorbic acid 0.2 in 2 mM phosphate buffer, pH 7.4. All chemicals were of analytical grade. Solvents were of HPLC grade. Microdialysis probes, CMA/12 (3 mm, 400 $\mu$m inner diameter (i.d.), 500 $\mu$m outer diameter (o.d.)) and CMA/20 (10 mm, 500 $\mu$m i.d., 670 $\mu$m o.d.), were supplied by CMA/Microdialysis (CMA, Solna, Sweden). The probe membranes have a 20 000 dalton molecular weight cutoff.

### *Surgical procedure*

The rats were kept under inhalation anaesthesia with 2% Enfluran and 1.5 l $min^{-1}$ nitrous oxide balanced with 1.5 l $min^{-1}$ oxygen during all surgical procedures. The 30 cm PE-50 cannulae were fused with 2 cm PE-10 cannulae and filled with saline containing 200 U heparin to prevent clotting. The PE-10 ends were inserted into the left femoral artery and vein in order to collect blood samples and to administer M3G and probenecid, respectively. The blood probe (CMA/20, 10 mm) was perfused with 0.1% low molecular weight heparin solution before it was inserted into the right jugular vein *via* a guide cannula and then fixed with two sutures. The anaesthetized rat was then placed on the stereotaxic instrument (David Kopf Instruments, Tujunga, U.S.A.) and a midline incision was made to expose the skull. A CMA/12 guide cannula was implanted into the striatum with the coordinates: lateral 2.7 mm, anterior 0.8 mm relative to the bregma, and ventral 3.8 mm relative to the brain surface. The guide cannula was fixed to the skull with a screw and dental cement, and the skin was sutured to cover this. The dummy probe was removed from the guide cannula and a CMA/12 (3 mm) probe was inserted into the striatum. A 20 cm piece of PE-50 tubing was looped subcutaneously distal to the posterior surface of the neck, allowing the perfusion solution to reach rat body temperature before it entered the brain probe. The protruding ends of all cannulae were passed subcutaneously to the posterior surface of the neck, and were protected by a plastic cap sutured to the skin. During the surgical procedure, the rat was placed on a heating pad to maintain a body temperature of 38°C. Animals were allowed to recover for 24 h before the start of the experiment.

### *Calculation of recovery* in vivo

The experiments were carried out over 2 days, and an *in vivo* recovery procedure was performed on each day before the start of the experiment. The probes were perfused with perfusion solution for 60 min to stabilize the system and to obtain blank samples. After the blank period, the probes were calibrated *in vivo* over 100 min using a perfusion solution containing 1 and 2 $\mu$M M3G for brain and blood probes, respectively, according to the retrodialysis-by-drug method (Bouw & Hammarlund-Udenaes, 1998). Thereafter, the perfusate was switched back to blank perfusion solution for the washout period of 60 min and throughout the rest of the experiment.

The probes were perfused using a CMA/100 Microinjection Pump (CMA, Solna, Sweden). The microdialysis samples were collected automatically by a CMA/140 Microfraction Collector (CMA, Solna, Sweden). *In vivo* recovery of M3G was estimated by the loss of M3G from the perfusate during the retrodialysis period (equation 1):

$$\text{Recovery}_{\text{in vivo}} = \frac{C_{in} - C_{out}}{C_{in}} \quad (1)$$

where $C_{in}$ is the M3G concentration in the incoming perfusate, and $C_{out}$ is the M3G concentration in the outgoing dialysate. The concentrations of unbound M3G in brain ECF and blood were calculated from the dialysate concentrations corrected by *in vivo* recovery.

### *Study design*

There were two groups of rats; the control group ($n=7$) received a 4 h infusion of M3G each day for 2 days, and the probenecid group ($n=7$) received two daily 4 h infusions of M3G and also received an infusion of probenecid on the second day. M3G was dissolved in saline and probenecid was dissolved in 5% sodium bicarbonate in saline. The solutions were stored at 4°C for a maximum of 2 weeks. In the control group, the experiment was repeated on the second day to investigate any effect of repeated administration on systemic clearance or transport of M3G across the BBB. On day 1, the rats received an exponential infusion of M3G over 4 h to rapidly reach the target plasma concentration of 65 $\mu$M (30 $\mu$g/ml), using the Stanpump CCI system (Shafer *et al*., 1988). No excitatory effects of M3G are present at 65 $\mu$M (Gårdmark *et al*., 1998). The pharmacokinetic parameters for Stanpump were taken from Ekblom *et al*. (1993). The rats were followed for another 2 h postinfusion. On day 2, the rats received the same treatment as on day 1, except that four of the rats were decapitated immediately after stopping the infusion. The brain tissue was cleaned of surface blood vessels, collected and frozen at −20°C until analysis. Each brain half was assayed separately to compare the total concentrations on the probe side *vs* the side not influenced by probe implantation.

In the probenecid group, the rats were constantly infused with 5% sodium bicarbonate in saline (vehicle of probenecid) throughout the experiment from the start of the blank period until stopping the M3G infusion on day 1. The flow rate and

volume of saline solution were the same during both days. The administration procedure of M3G was the same as for the control group. On day 2 the rats received an intravenous loading dose of probenecid (70 $\mu$mol kg$^{-1}$) at the start of the blank period, followed by a constant infusion of 70 $\mu$mol kg$^{-1}$ h$^{-1}$ with a flow rate of 1.3 $\mu$mol kg$^{-1}$ h$^{-1}$, continued throughout the experiment (total infusion duration was about 8 h). The dose of probenecid was based on work by Wong *et al*. (1993). The rats received the same dose of M3G as on day 1, and four of them were subsequently decapitated and the brain tissue treated as mentioned above.

For both groups, microdialysis samples were collected at 20 min intervals with a perfusion flow rate of 1 $\mu$l min$^{-1}$ and these were frozen at $-20^{\circ}$C until analysis. Arterial blood (100 $\mu$l) was drawn at 0, 10, 60, 120, 180, 240, 245, 250, 270, 300 and 360 min. The plasma was separated by centrifugation (10,000 r.p.m., 5 min) and frozen at $-20^{\circ}$C until analysis.

### *Sample analysis*

*Microdialysis samples* Seventeen $\mu$l of the microdialysis samples were directly injected into the Nucleosil $C_{18}$ high performance liquid chromatography (HPLC) column (5 $\mu$m particles, 4.6 × 150 mm, The Netherlands). M3G was analysed by fluorescence detection (Jasco 821-FP, Japan) at an excitation wavelength of 212 nm and an emission wavelength of 340 nm. The flow rate was 1 ml min$^{-1}$. For brain microdialysis samples, the mobile phase consisted of 650 ml 0.01 M phosphate buffer (pH 2.1) containing 0.4 mM sodium dodecyl sulphate (SDS), 350 ml methanol and 20 ml tetrahydrofuran. For blood microdialysis samples, the mobile phase consisted of 650 ml 0.01 M phosphate buffer (pH 2.1) containing 0.4 mM SDS and 350 ml methanol. The limit of quantification of M3G in microdialysis samples was 170 nM, with a coefficient of variation (CV) of 6.8%.

*Plasma and brain samples* The plasma samples of M3G were extracted with Sep-Pak $C_{18}$ cartridges (Waters), which were first activated with 5 ml methanol, 3 ml 0.01 M phosphate buffer (pH 2.1) and 5 ml distilled water filtered through the cartridge under vacuum in order. Plasma (diluted with blank plasma to 100 $\mu$l) was mixed with 3 ml of 0.5 M ammonium sulphate buffer (pH 9.3) in a 10 ml polystyrene tube for 5 s, and transferred to the reservoir. The plasma samples were filtered through the cartridges, which were subsequently washed with 20 ml 5 mM ammonium sulphate buffer (pH 9.3), 0.5 ml distilled water, and 0.1 ml methanol under vacuum. Lastly, 3 ml methanol was added and the eluates were collected and evaporated under a stream of nitrogen at 45°C. The dried residues were redissolved in 150 $\mu$l mobile phase, of which 50 $\mu$l was injected onto the HPLC column. M3G was analysed by fluorescence detection (Jasco 821-FP, Japan) at an excitation wavelength of 212 nm and an emission wavelength of 340 nm. The mobile phase consisted of 670 ml 0.01 M phosphate buffer (pH 2.1) containing 0.2 mM SDS, 330 ml methanol and 50 ml tetrahydrofuran. The limit of quantification of M3G in plasma was 0.11 $\mu$M, with a CV of 9–12%. The absolute extraction recovery for plasma samples of M3G was 100 ± 2%.

One half of the whole brain tissue was homogenized with a 5 fold volume of 0.1 M perchloric acid and then centrifuged for 20 min at 5000 r.p.m. at 4°C. Four hundred $\mu$l of the supernatant were extracted in the same way as the plasma samples. The analytical method for brain tissue was the same as for plasma, except for a mobile phase consisting of 670 ml 0.01 M phosphate buffer (pH 2.1) containing 0.4 mM SDS, 330 ml methanol and 20 ml tetrahydrofuran. The limit of quantification of M3G in brain tissue samples was 0.21 $\mu$M, with a CV of 7.0%. The absolute recovery of M3G from brain tissue was 98 ± 3%.

### *Data analysis*

Body clearance was calculated using a non-compartmental method according to the standard procedure ($CL_u$ = Dose/AUC) for individual rats, where AUC stands for area under the unbound plasma concentration-time curve. The terminal half-lives of M3G in brain ECF and blood were estimated by log-linear regression of the last 3–4 observations. The extent of the BBB transport of M3G was calculated as the unbound steady state concentration ratio of brain ECF to blood ($C_{u,ss,br}/C_{u,ss,bl}$).

### *Development of a distributional model*

The pharmacokinetic model, which includes the arterial, venous and brain ECF distribution of M3G, is shown in Figure 1. The arterial plasma concentration is the driving force for drug transport into the brain. As the venous concentration-time profile contained more data, a model containing both arterial and venous concentration data strengthened the prediction of the pharmacokinetic parameters.

The models were analysed by non-linear regression, which was performed using non-linear mixed effect modelling with NONMEM (version VI), first-order (OF) method (Model A and B) and first-order conditional estimation (FOCE), method (Model B) (Beal & Sheiner, 1992). First, all of the arterial and venous concentrations were simultaneously fitted to Model A, obtaining pharmacokinetic parameters. As the volume of distribution in the venous compartment could not be determined from the model, the volume was fixed as 1, making the clearance from the venous compartment equal to the rate constant. The protein binding of M3G in plasma is very low (7%) (Bickel *et al*., 1996) and was therefore neglected. In a second step (Model B), the individual plasma concentration parameters (defined by the individual estimates obtained from the first step) were used as a forcing function in the fit of brain ECF concentrations to predict the BBB transport parameters. The concentrations of M3G in brain ECF in the control and probenecid groups were fitted simultaneously. The mass change of drug in the arterial compartment due to transfer into and out of the brain was ignored. One- and two-compartment brain models were tested, and a two-compartment model was found to best describe the distribution of M3G in the brain. The model selection is described below. The two compartments were defined as brain 1 and brain 2 compartments.

The differential equation for the distribution of M3G in brain 1 is expressed by equation 2 (Model B).

$$V_{u,\,br1} * \frac{dC_{u,\,br1}}{dt} = CL_{u,\,in} * C_{u,\,pl} + Q_{br} * C_{u,\,br2} - (CL_{u,\,out} + Q_{br}) * C_{u,\,br1} \quad (2)$$

where $C_{u,pl}$ is the arterial plasma concentration of M3G. $C_{u,br1}$ and $C_{u,br2}$ are the concentrations of unbound M3G in the brain 1 and brain 2 compartments, respectively. $CL_{u,in}$ and $CL_{u,out}$ are the influx and efflux clearances into and from brain 1, respectively. Distribution between brain 1 and brain

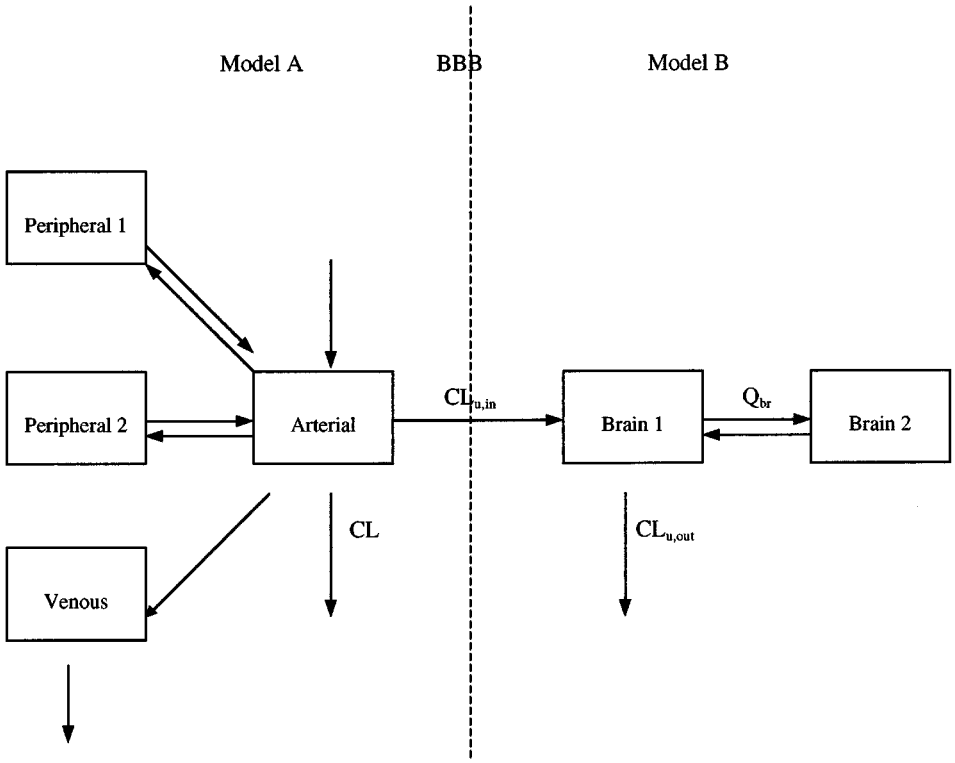


**Figure 1** The arterial plasma and venous blood relationship is described in Model A. The pharmacokinetic distributional model (Model B) illustrates unbound drug transport across the BBB and equilibration between brain 1 and brain 2 compartments.

2 compartments was characterized by the intercompartmental clearance $Q_{br}$. $V_{u,br1}$ is the volume of distribution in the brain 1 compartment. The apparent unbound volume of distribution ($V_{u,app}$) in the brain was calculated using equation 3 (Wang & Welty, 1996), and the volume of distribution in the brain 2 compartment ($V_{u,br2}$) is equal to ($V_{u,app} - V_{u,br1}$).

$$V_{u,\,app} = \frac{(A_{br} - V_{bl} * C_{bl})}{C_{u,\,br1}} \quad (3)$$

where $A_{br}$ is the total amount of M3G per g-brain. $V_{bl}$ and $C_{bl}$ are the volume of blood in one gram of brain and the M3G concentration in arterial blood, respectively. The blood-to-plasma ratio of M3G at a haematocrit of 44% was taken as 0.53 (Skopp *et al.*, 1998), and blood in the brain tissue is about 1–2% of the brain weight (Rosenberg, 1990). The plasma space in the brain is $14 \pm 1$ $\mu$l g-brain$^{-1}$ (Bickel *et al.*, 1996). Based on this, $V_{bl}$ is taken to be 15 $\mu$l g-brain$^{-1}$ in the calculation of $V_{u,app}$. In the regression analysis, $V_{u,app}$ was fixed to the value that was estimated from equation 3. Equation 2 was re-parameterized in terms of ratio $CL_{u,in}$ and $CL_{u,out}$ (Ratio = $CL_{u,in}/CL_{u,out}$) (equation 4) in order to obtain the relative standard error (RSE) for this parameter.

$$V_{u,\,br1} * \frac{dC_{u,\,br1}}{dt} = CL_{u,\,in} * C_{u,\,pl} + Q_{br} * C_{u,\,br2} - \left(\frac{CL_{u,\,in}}{Ratio} + Q_{br}\right) * C_{u,\,br1} \quad (4)$$

To test whether the BBB transport parameters were influenced by probenecid, the different models were examined with the same or different parameters in the presence (probenecid group on day 2) and absence (control and probenecid groups on day 1) of probenecid, respectively. For instance, one model was set with different influx clearances for the presence and absence of probenecid, and another model was set with the same value for influx clearance for both situations. The model selection was guided by the decrease in objective function value ($-2$ times the log likelihood value), as well as by graphical analyses of residuals and predictions with Xpose 2.0 (Jonsson & Karlsson, 1999). The adjustment of the statistical significance of additional parameters is based on the difference between the objective function values between two hierarchical models, with corresponding values of 11 and 14 for the 0.1% level at 1 and 2 degrees of freedom for sparse observations. It is difficult to determine the level of significance for rich data (as obtained in the present study) using the sparse data obtained from the model, but the level is higher for rich data (Karlsson, personal communication). An exponential variance model was used to describe the inter-individual variability (equation 5), where $P_i$ and $P_{pop}$ are the parameters for the ith ($i = 1,...n$) subject and the average population estimates, respectively. $\eta_i$ is a zero mean and normally distributed variable with standard deviation $\omega$, which has been estimated.

$$P_i = P_{pop} * \exp(\eta_i) \quad (5)$$

The residual error model for the observed concentrations ($Y_{obs}$) was characterized by a proportional error model described by equation 6, where $Y_{pred}$ is the predicted concentration, and $\varepsilon$ is a zero-mean normally distributed variable with standard deviation $\sigma$.

$$Y_{obs} = Y_{pred} * (1 + \varepsilon) \quad (6)$$

The basic factors of the graphical expression are observed concentrations and model predictions based on the population parameters, and individual predictions based on *post-hoc* Bayesian estimates.

### *Statistical analysis*

Repeated measures ANOVA (StatView 4.5) were used to compare parameter differences between groups (between

factors) and days (within factors). If significant difference was found from ANOVA main table, further paired *t*-test (within group) and unpaired *t*-test (between groups) were performed. The level of statistical significance was $P<0.05$. The data were presented as means$\pm$s.d. The precision of the estimation of the typical parameters from the NONMEM output was expressed as the relative standard error (RSE).

## Results

The *in vivo* recovery of M3G from both the blood and the brain probes did not vary significantly between the two experimental days in the control group (Table 1). During probenecid treatment on day 2 in the treatment group, however, recovery of M3G from the brain probes (6.2%) was significantly lower than during day 1 (9.1%) ($P<0.001$). There was no statistically significant difference in recovery of the blood probe between days in the probenecid group ($P=0.08$).

M3G slowly reached BBB equilibrium (Figure 2). M3G was transported into brain ECF to only a low extent (Figure 2). The ratio of the unbound M3G steady-state concentration in brain to that in blood in the control group was the same for day 1 and day 2 (0.11 and 0.10, respectively (Table 2); the ratio of the value for day 2 to that for day 1 was $0.98\pm0.35$). In the probenecid group, the unbound M3G concentration in brain ECF during probenecid co-administration was twice as high as without probenecid (Figure 2b). The ratio of the unbound concentration of M3G in brain to that in blood on day 2 in the probenecid group (0.16) was significantly higher than on day 1 (0.08, $P=0.001$; Table 2), and also significantly higher than the average ratio in the combined control and probenecid groups on day 1 ($0.10\pm0.04$, $P=0.018$). The day 2 to day 1 ratio was $1.96\pm0.25$ in the probenecid group. Although higher, the unbound M3G concentration-time profiles in brain ECF has similar shapes and half-lives with or without concomitant probenecid (Figure 2, Table 2). The average half-life in brain ECF ($81\pm25$ min) was much longer than in blood ($22\pm2$ min). There is no significant difference in blood concentration-time profiles and pharmacokinetics of M3G in the absence or presence of probenecid.

The total brain concentrations of M3G were $2655\pm979$ nM g-brain$^{-1}$ in the side of the brain containing the probe and $2590\pm1361$ nM g-brain$^{-1}$ in the other half (n.s.). The apparent volume of distribution of unbound M3G in brain was $0.25\pm0.02$ ml g-brain$^{-1}$ and $0.21\pm0.11$ ml g-brain$^{-1}$ (n.s.) for the control and probenecid groups, respectively. This results in higher unbound brain ECF concentration than total brain tissue concentration.

The clearance and half-life of unbound M3G in plasma, blood and brain ECF were similar on both days and in both groups (Table 2). The average clearance of unbound M3G was $3.72\pm0.65$ ml min$^{-1}$ and $4.46\pm0.57$ ml min$^{-1}$ (n.s.) for arterial plasma and venous blood, respectively. The arterial-venous concentration Model A (Figure 1) adequately described the concentrations of plasma and blood simultaneously, resulting in an unbound clearance of 3.8 ml min$^{-1}$. In the BBB distributional model analysis, the objective function for the model with differing values for $CL_{u,in}$ in the presence and absence of probenecid was lower than that for the model with the same values for $CL_{u,in}$ by 448 units ($P<0.001$). Then the model with changing $CL_{u,out}$ was compared to that with no change, the difference between the objective functions was 14. The overall fit to the data was improved with a smaller trend in the residuals in the model

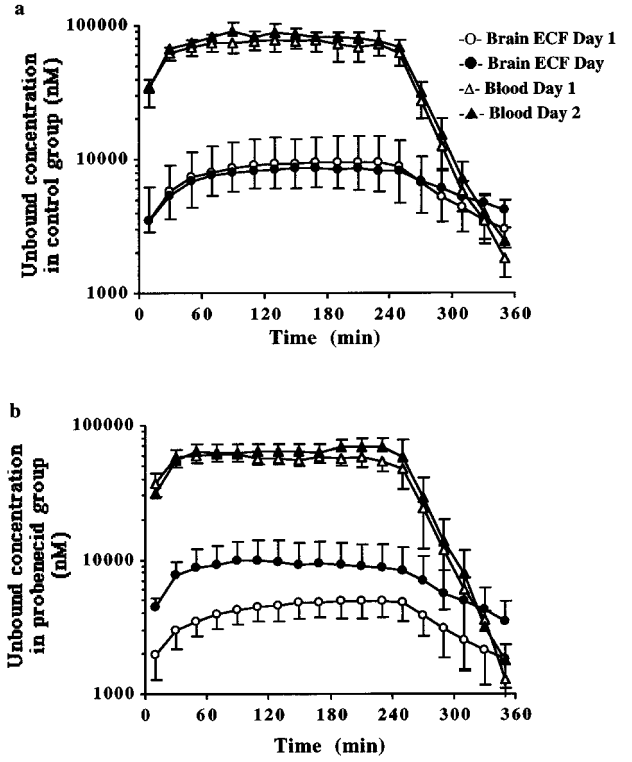


**Figure 2** Concentration-time profiles of unbound M3G during exponential i.v. infusion over 4 h and 2 h after infusion in the control group (a), and probenecid group (b). Data are presented as means$\pm$s.d.

**Table 1** *In vivo* recovery (retrodialysis loss) of unbound M3G on days 1 and 2 in brain and blood of rats receiving M3G alone or with probenecid (mean$\pm$s.d.)

| *Probe* (n=7) | *Control group* RD%$_{D1}$# | RD%$_{D2}$§ | *Probenecid group* RD%$_{D1}$† | RD%$_{D2}$†† |
|---|---|---|---|---|
| Blood (10 mm) | $39.5\pm11.6$ | $36.7\pm5.9$ | $46.8\pm14.5$ | $35.2\pm9.1$ |
| Brain (3 mm) | $8.2\pm1.9$ | $8.5\pm2.7$ | $9.1\pm1.4$ | $6.2\pm1.1$* |

#Retrodialysis loss during day 1 in the control group.
§Retrodialysis loss during day 2 in the control group.
†Retrodialysis loss during day 1 in the probenecid group.
††Retrodialysis loss during day 2 in the probenecid group.
*Statistically significant difference from day 1 ($P=0.0001$, paired *t*-test).

with differing $CL_{u,in}$, but there was no improvement for the model with differing $CL_{u,out}$ or in the models with differing $V_{br}$ and $Q_{br}$. The same result was found for the model in which the ratios of $CL_{u,in}/CL_{u,out}$ differed; the objective function value decreased by 436 ($P<0.001$).

Consequently, the final choice was a two-compartment model which included different influx clearances (or ratios) but otherwise had the same parameters for the presence and absence of probenecid (equation 2 or 4), since this model described the data the best (Figure 3). The influx clearance into the brain in the presence of probenecid was 1.5 fold higher than that in the absence of probenecid (i.e. in the combined control and probenecid groups on day 1; Table 3). The intercompartmental clearance was 0.95 $\mu$l min$^{-1}$ g-brain$^{-1}$, and the volumes of distribution for the brain 1 and brain 2 compartments were 28 $\mu$l g-brain$^{-1}$ and 205 $\mu$l g-brain$^{-1}$, respectively. The ratios of $CL_{u,in}/CL_{u,out}$ in the

presence and absence of probenecid (Table 3) were similar to the ratio of the unbound M3G steady-state concentration in brain ECF to that in blood (Table 2).

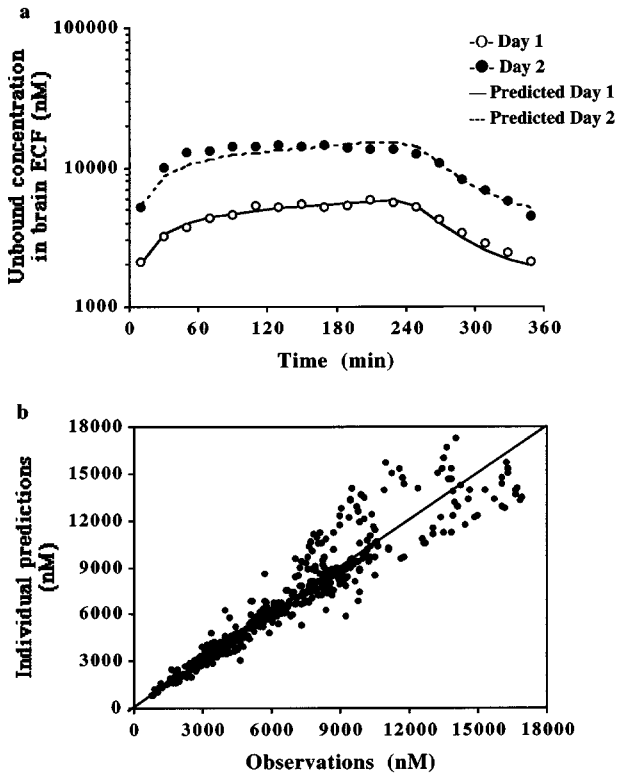


**Figure 3** (a) Actual concentrations of unbound M3G *vs* time in brain ECF of one rat in the probenecid group, with predictions (Model B), and (b) individual predictions *vs* actual concentrations for the control and probenecid groups, based on the Bayesian estimates of individual parameters, during exponential i.v. infusion for 4 h and 2 h after infusion. Straight line is line of identity.

## Discussion

This study was designed to investigate the influence of probenecid on the transport of M3G across the BBB. The influx clearance of M3G estimated in this study (0.11 $\mu$l min$^{-1}$ g-brain$^{-1}$) was close to the product of permeability and surface area (PS) (0.14 $\mu$l min$^{-1}$ g-brain$^{-1}$) (Bickel *et al*., 1996). The brain ECF M3G concentration was doubled in the presence of probenecid, indicating the involvement of a probenecid-sensitive organic anion transport system.

The *in vivo* recovery of M3G is an important issue in correcting the concentration obtained from dialysis to the true concentration in the tissue. For both types of probes, there were no statistical differences in *in vivo* recoveries for the control group between the two experimental days, suggesting a stable system during the experimental period with time-independent *in vivo* recovery. The significantly lower *in vivo* recovery of M3G observed for the striatal probes when probenecid was co-administered indicates that recovery is sensitive to the active processes in the brain tissue, since blood probes did not show differences in recovery with concomitant probenecid. It has also been reported that the *in vivo* recovery of rhodamine-123 and morphine is lower in *mdr1a* (−/−) mice than in *mdr1a* (+/+) mice (de Lange *et al*., 1998; Xie *et al*., 1999), and the *in vivo* recovery of caffeine is decreased with concomitant adenine, an inhibitor of active transport (Song & Lunte, 1999). These experiments all tend to confirm the theory that recovery is dependent on tissue transport (Bungay *et al*., 1990). The only difference between the 2 days was the presence of probenecid. Thus, the probenecid-dependent extracellular-microvascular exchange must have been influenced. The presence of the inhibitor of active transport, probenecid, would increase resistance to mass transfer of M3G from the ECF to the probe, resulting in lower *in vivo* recovery than when probenecid was absent.

The fact that the ratio of the steady-state unbound M3G concentration in brain ECF to that in blood was similar for

**Table 2** Noncompartmental pharmacokinetic parameters for M3G in rats (mean ± s.d.)

| | *Control group* | | *Probenecid group* | |
|---|---|---|---|---|
| | *Day 1* | *Day 2* | *Day 1* | *Day 2* |
| $CL_{pl}$ # (ml min$^{-1}$) | 4.48 ± 0.62 | 3.85 ± 0.04 | 4.90 ± 0.26 | 4.12 ± 0.49 |
| $CL_{bl}$ § (ml min$^{-1}$) | 3.38 ± 0.44 | 3.15 ± 0.46 | 4.29 ± 0.48 | 3.92 ± 0.98 |
| $t_{1/2,bl}$ † (min) | 22.2 ± 2.1 | 21.9 ± 1.6 | 21.4 ± 3.9 | 20.0 ± 1.1 |
| $t_{1/2,br}$ †† (min) | 70.1 ± 14.5 | 107.8 ± 26.2 | 77.7 ± 23.8 | 86.7 ± 16.6 |
| $C_{u,ss,br}/C_{u,ss,bl}$ ## | 0.11 ± 0.05 | 0.10 ± 0.02 | 0.08 ± 0.02 | 0.16 ± 0.05* |

#Clearance calculated from arterial plasma ($n=7$ and 3 on days 1 and 2, respectively). §Clearance calculated from venous blood with microdialysis. †Terminal half-life of M3G in venous blood. ††Terminal half-life of M3G in brain ECF. ##The ratio of the unbound M3G steady-state concentration in brain ECF ($C_{u,ss,br}$) to that in blood ($C_{u,ss,bl}$). *Significant difference from control group day 1 ($P=0.038$, unpaired *t*-test), day 2 ($P=0.03$, unpaired *t*-test), and probenecid group on day 1 ($P=0.004$, paired *t-test*).

**Table 3** Parameter estimates (RSE%) for the BBB transport of M3G using Model B

| | *Without probenecid#* | | *With probenecid §* | |
|---|---|---|---|---|
| *Parameter* | *Estimate* | *IAV*** | *Estimate* | *IAV*** |
| $CL_{u,in}$ ($\mu$l min$^{-1}$ g-brain$^{-1}$)† | 0.11 (25) | 49 (25) | 0.17 (25)* | 20 (47) |
| $CL_{u,out}$ ($\mu$l min$^{-1}$ g-brain$^{-1}$)†† | 1.15 (34) | 50 (65) | 1.15 (34) | 50 (65) |
| $CL_{u,in}/CL_{u,out}$ ## | 0.09 (13) | 50 (25) | 0.14 (19)* | 48 (53) |
| Residual variability | 0.14 (20) | NA | | |

#Control group plus probenecid group on day 1. §Probenecid group on day 2. †The influx clearance into brain ECF. ††The efflux clearance out of brain ECF, set as one parameter for both with and without probenecid. ##The ratio of influx clearance to efflux clearance, estimated using equation 4. **Interanimal variability (%). *Significant difference from without probenecid ($P<0.001$).

both experimental days in the control group indicates that the BBB transport of M3G was independent of time and that the 2 day study design was a valid choice. The pharmacokinetics of M3G on day 1 for both control (without 5% bicarbonate) and probenecid (with 5% bicarbonate) groups were similar. There was no difference in concentration ratio of brain ECF to blood between the two groups on day 1 ($P=0.61$, unpaired $t$-test). Thus, the pharmacokinetics and BBB transport of M3G were not affected by presence of 5% bicarbonate alone.

The steady-state concentration of unbound M3G in brain ECF doubled after probenecid treatment. It was also shown in the model analysis that the ratio of influx clearance to efflux clearance increased 1.6 fold in the presence of probenecid. This suggests that a probenecid-sensitive organic anion transport system is involved in the BBB transport of M3G. Previous studies have indicated that the BBB transport of M3G is not affected by Pgp (Xie *et al*., 1999; Letrent *et al*., 1999). Unlike Pgp, a family of multidrug resistance proteins (MRPs) can actively transport a range of substrates that are conjugated to glutathione, glucuronide or sulphate (Keppler *et al*., 1998). The MRPs characterized thus far are all organic anion transporters (Borst *et al*., 1999). It has also been demonstrated that probenecid can reverse the effects of the MRPs (Hooijberg *et al*., 1999). It has been found that the *MRP* gene is expressed in human brain tissue (Kool *et al*., 1997), rat brain tissue and cell lines (Regina *et al*., 1998), mouse brain cell lines (Kusuhara *et al*., 1998), and bovine brain microvessel endothelial cells (Huai-Yun *et al*., 1998). It is possible, therefore, that the probenecid-sensitive transporter of M3G in rat brain is an MRP.

The pharmacokinetics of M3G in blood was not significantly influenced by the presence of probenecid at the given dose (clearance and half-life were not changed). Thus, since the transport across the BBB was influenced without affecting the blood concentration-time profile, it appears that the transporter acting on M3G is specific for the BBB or that the effect of this transporter on the peripheral elimination of M3G (e.g. in bile or urine) is too small to statistically affect the systemic pharmacokinetics. In contrast to our findings, it has been reported that both blood and brain pharmacokinetics of zidovudine are affected by probenecid (Wong *et al*., 1993). It has also been shown that the transport of several drugs, such as baclofen, quinolinic acid, 17$\beta$-estradol 17$\beta$-D-glucuronide and 1-naphthyl-$\beta$-D-glucuronide, across the BBB is sensitive to the presence of probenecid (Deguchi *et al*., 1995; Morrison *et al*., 1999; Nishino *et al*., 1999; Strazielle & Ghersi-Egea, 1999).

The ratio of the steady-state concentration of unbound M3G in brain ECF to that in blood was much less than unity, even after probenecid treatment (16%). The interstitial bulk flow in rat brain is 0.18–0.29 $\mu$l min$^{-1}$ g-brain$^{-1}$ (Szentistvanyi *et al*., 1984). The efflux clearance (1.15 $\mu$l min$^{-1}$ g-brain$^{-1}$) was greater than the interstitial bulk flow. The contribution of bulk flow to efflux clearance was 16–25%, indicating that it might play a small role in M3G elimination. This suggests that the transporter is not fully blocked by probenecid at this dose and/or that there are other active transporters acting on M3G. It has been reported that very high concentrations of inhibitor are needed to completely block the active process (Golden & Pollack, 1998), and that inhibitors generally only partially block MRP (Aszalos & Ross, 1998).

The actual location of probenecid-sensitive organic anion transport systems in the BBB, i.e. on the luminal or the abluminal side, has not been identified. It is unknown which BBB transport processes would be affected: influx into or efflux out of the brain, or both. The findings in this paper support the hypothesis that the probenecid-sensitive transport system affects the influx clearance of molecules, since the half-lives and general pattern of the concentration-time profiles for M3G were similar for both study groups. The presence of probenecid raise the concentration-time curves, indicating that the rate of infusion of M3G into the brain was increased. The influx clearance into the brain was increased from 0.11 to 0.17 $\mu$l min$^{-1}$g-brain$^{-1}$ after probenecid administration, and analysis of the model indicated a better fit with this scenario. This implies that there is a significant difference between influx clearances in the two model situations, and that probenecid will influence the influx clearance to a greater extent than the efflux clearance.

Probenecid-sensitive transport of M3G at the BBB could be likened to a 'gate keeper' effect, hindering M3G transport into the brain. However, it is possible that changed efflux clearance was not observed/predicted because redistribution within the brain was the rate-limiting step. It has been recently shown by Homma *et al*. (1999) that the efflux transporter system for glutathione bimane (GS-B) occurs on both the luminal and abluminal sides of MBEC4 (mouse brain capillary endothelial cell line) monolayers, but the affinity of the efflux transport systems to GS-B on the luminal membrane was considered to be higher than that on the abluminal membrane. It was suggested that an energy-dependent efflux transport system for organic anions is located on the luminal membrane of the MBEC4 monolayers.

Since the half-life of M3G is 81 min in the brain and 22 min in blood, and since the model required two compartments, it would appear that the redistribution process within the brain tissue, rather than transport across the BBB itself, is the rate-limiting step for the elimination of M3G from the brain. However, the concentration-time profiles in brain ECF and blood will eventually become parallel due to redistribution. In the modelling analysis we assumed that the influence of the concentration of M3G in the brain on that in the blood was negligible. This assumption was valid, since the amount in brain was about 0.02% of the amount in the rest of the body at steady-state.

Extracellular space accounts for about 15–20% of the brain tissue (Rosenberg, 1990). The volume of distribution of M3G (0.23 ml g-brain$^{-1}$) was larger than the rat brain inulin extracellular space of 0.14 ml g-brain$^{-1}$ (Woodward *et al*., 1967), but was close to the ion volume fraction of 0.21 (cations tetramethyl ammonium and anions $\alpha$-naphthalene sulphonate, etc.) (Nicholson & Phillips, 1981). This suggests that M3G does not bind to or accumulate in the brain tissue, but is mainly distributed throughout the interstitial space. In spite of this, previous workers have found that the transport of gabapentin across intracellular and interstitial membranes in brain tissue is much greater than that across the BBB, with a volume of distribution in the brain of 5.5 ml g-brain$^{-1}$ (Wang & Welty, 1996). This resulted in the choice of a one-compartment model for the distribution of gabapentin in the brain. The more hydrophilic nature of M3G is presumed to account for the different results of our study.

While the ratio of the unbound M3G steady-state concentration in brain to that in blood was 0.08 to 0.11 in this study, the ratio of the total M3G was 0.04. The latter value fits well with our previous report in mice of 0.05 (Xie *et al*., 1999) and in rats of 0.02 (Murphey & Olsen, 1994), but not with the ratio of 0.68 found by Letrent *et al*. (1999). The higher ratio for unbound drug than for total drug may have occurred because M3G does not penetrate well into cells,

resulting in a smaller unbound volume of distribution and lower total brain concentration compared to unbound concentration. If this was the case, the brain homogenate method would give a different answer from that obtained by microdialysis; microdialysis describes the BBB transport process while the homogenate method includes the total accumulation including binding within the brain tissue.

In summary, while the concentration of M3G in the brain slowly reached equilibrium, the concentration of unbound M3G in brain ECF was very low. Probenecid increased the concentration of unbound M3G in brain ECF without influencing the blood concentrations, which demonstrates that a probenecid-sensitive transport system, possibly MRP, is involved in the transport of M3G across the BBB. The distributional model describing the effect of probenecid on the BBB transport of M3G suggested that the probenecid-sensitive transporter has the greatest impact on the influx clearance of M3G and that this transporter is specific for the BBB. Microdialysis provides insight into the equilibration of unbound drugs across the BBB. We conclude that microdialysis is a very good method for the quantification of influx and efflux clearances across the BBB, without the confounding influence of drug binding to brain or blood components.

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