Morphine Brain Pharmacokinetics at Very Low Concentrations Studied with Accelerator Mass Spectrometry and Liquid Chromatography-Tandem Mass Spectrometry

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ABSTRACT:

Morphine has been predicted to show nonlinear blood-brain barrier transport at lower concentrations. In this study, we investigated the possibility of separating active influx of morphine from its efflux by using very low morphine concentrations and compared accelerator mass spectrometry (AMS) with liquid chromatography-tandem mass spectrometry (LC-MS/MS) as a method for analyzing microdialysis samples. A 10-min bolus infusion of morphine, followed by a constant-rate infusion, was given to male rats (n = 6) to achieve high (250 ng/ml), medium (50 ng/ml), and low (10 ng/ml) steady-state plasma concentrations. An additional rat received infusions to achieve low (10 ng/ml), very low (2 ng/ml), and ultralow (0.4 ng/ml) concentrations. Unbound morphine concentrations from brain extracellular fluid and blood were sampled by microdialysis and analyzed by LC-MS/MS and AMS. The average partition coefficient for unbound drug (K_p,uu) values for the low and medium steady-state levels were 0.22 ± 0.08 and 0.21 ± 0.05, respectively, when measured by AMS [not significant (NS); p = 0.5]. For the medium and high steady-state levels, K_p,uu values were 0.24 ± 0.05 and 0.26 ± 0.05, respectively, when measured by LC-MS/MS (NS; p = 0.2). For the low, very low, and ultralow steady-state levels, K_p,uu values were 0.16 ± 0.01, 0.16 ± 0.02, and 0.18 ± 0.03, respectively, when measured by AMS. The medium-concentration K_p,uu values were, on average, 16% lower when measured by AMS than by LC-MS/MS. There were no significant changes in K_p,uu over a 625-fold concentration range (0.4–250 ng/ml). It was not possible to separate active uptake transport from active efflux using these low concentrations. The two analytical methods provided indistinguishable results for plasma concentrations but differed by up to 38% for microdialysis samples; however, this difference did not affect our conclusions.

Introduction

To enter the central nervous system (CNS) via the blood, a drug must pass the blood-brain barrier (BBB), which consists of endothelial cells connected by tight junctions. The exposure of the brain to unbound drug is determined by the chemical structure of the drug and any active processes, either influx or efflux, at the BBB will significantly affect the concentration of drugs in the brain and, hence, modify the central therapeutic effects.

Morphine, one of the most commonly used analgesics in terminal illness or for moderate to severe pain, acts on the CNS. This drug is a substrate for nonspecific probenecid-sensitive transporters. Oral pretreatment of rats with the specific P-glycoprotein (Pgp) inhibitor N-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinoliny1)-ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide (GF120918) caused a prolonged morphine-associated antinociceptive effect due to morphine’s prolonged half-life in the brain (Tunblad et al., 2003). In one study, coadministration of probenecid decreased the brain efflux of morphine, thus increasing the K_p,uu from 0.29 to 0.39 (Tunblad et al., 2003). This result indicates that morphine is a substrate for nonspecific probenecid-sensitive transporters.

ABBREVIATIONS: CNS, central nervous system; BBB, blood-brain barrier; ISF, interstitial fluid; K_p,uu, partition coefficient for unbound drug; Pgp, P-glycoprotein; GF120918, N-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinoliny1)-ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide; AMS, accelerator mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; ACN, acetonitrile; TFA, trifluoroacetic acid; D_3, deuterated; LC, liquid chromatography V_blood, volume of blood per g brain.
BBB transport of morphine in sheep differs from that in other species. There is a clear active influx of morphine, with $K_{pu,m}$ values of 1.89 in premature lambs and 1.19 in adult sheep (Bengtsson et al., 2009). The first evidence of the active influx of an opioid in any species was the three-times-higher concentration of unbound oxycodeine in the rat brain than in blood (i.e., $K_{pu,m} = 3$) (Boström et al., 2006). This result is in sharp contrast to the net active efflux of morphine across the BBB, although morphine and oxycodeine have similar structures and similar lipophilic properties (Boström et al., 2006). There are also studies indicating that morphine is actively transported into the brain in some species. Xie et al. (1999) found that, in mice, there was a tendency toward a higher $K_{pu,m}$ at low plasma concentrations. This result indicates that the transport of morphine into the human brain might occur not only by passive diffusion but also by active influx. It has been suggested that the mechanism for active uptake of morphine into the brain could involve low-capacity active influx, which is saturated at low concentrations and therefore results in a net efflux across the BBB at higher morphine concentrations (Groenendaal et al., 2007). The influx transporter of oxycodeone and morphine is likely to be the same, but because it has not yet been characterized (Okura et al., 2008), there is neither a specific inhibitor nor a knockout animal for use in studies.

Another problem associated with studying low-capacity transport is the need for a method of chemical analysis that is capable of detecting very low concentrations of morphine. Because of its very high sensitivity, accelerator mass spectrometry (AMS) has been used in many fields, including archeology, anthropology, space and earth sciences, environmental sciences, and new drug development (Garner et al., 2000). AMS is many orders of magnitude more sensitive than conventional methods because of its capacity for directly counting the $^{14}$C atoms instead of monitoring radioactive decay (Garner et al., 2000).

This study was designed to investigate the possibility of separating the active influx of morphine from the net efflux by using very low drug concentrations. The problem of analyzing such low concentrations was addressed by using AMS in combination with liquid chromatography-tandem mass spectrometry (LC-MS/MS). A second aim of this study was to validate AMS as a method for the analysis of microdialysis samples in comparison with LC-MS/MS.

Materials and Methods

Chemicals. $^{14}$C-labeled morphine was obtained from American Radiolabeled Chemicals (St. Louis, MO). Morphine hydrochloride ampoules (10 mg/ml Morfin Meda) were obtained from Apoteket (Uppsala, Sweden). Isoflurane Baxter was purchased from Baxter Medical AB (Kista, Sweden). Ammonium acetate, acetoniirile (ACN), and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). Deuterated (D$_3$)-morphine hexahydrate and $^{14}$C-labeled morphine were obtained from American Radiolabeled Chemicals. Ringer’s solution (R-CNS) consisted of 147 mM NaCl, 2.7 mM KCl, 1.2 mM CaCl$_2$, and 0.85 mM MgCl$_2$ in purified water using a Milli-Q academic system (Millipore Corporation, Billerica, MA). Physiological saline was purchased from Braun Medical AB (Stockholm, Sweden).

Animals. Seven male Sprague-Dawley rats (obtained from B&K, Sollentuna, Sweden) were used in the study. The animals were allowed to aclimatize to a 12-h light/dark cycle at 22°C for at least 7 days before the study and weighed between 260 and 300 g on the day of the experiment. The study was approved by the Animal Ethics Committee, Tierp District Court, Tierp, Sweden (approval number C2/8).

Animal Surgery. The rats ($n = 7$) were anesthetized with Isofluran-Baxter (2.5%) balanced with oxygen (1.5 l/min) and nitrous oxide (1.5 l/min). A heating pad with a temperature gauge was used to maintain the body temperature of the rats at 38°C during surgery. A PE-50 cannula fused to silastic PE-10 tubing was inserted into the left femoral vein for administration of morphine, and another PE-50 cannula fused to PE-10 tubing was inserted into the left femoral artery for blood sampling. To prevent clotting, the catheters were flushed with saline solution containing heparin 100 IE/ml (Heparin L01, 5000 IE/ml; Leo Pharma AB, Malmö, Sweden). A CMA/20 (10-mm) probe (CMA Microdialysis, Solna, Sweden) was inserted into the right jugular vein through a guide cannula and fixed with two stitches to the pectoral muscle. Each rat was placed in a stereotaxic instrument (David Kopf Instruments, Tujunga, CA), and the skull was exposed by making a midline incision. A hole was drilled 2.7 mm lateral and 0.8 mm anterior to the bregma and 3.8 mm ventral to the surface of the brain. A CMA/12 guide cannula was implanted into the striatum and fixed to the skull by a screw and dental cement (Dentalon Plus; Heraeus, Hanau, Germany). A CMA/12 (3-mm) probe was inserted after fixing the guide cannula. To allow the perfusion solution to adjust to body temperature before entering the brain probe, a 15-cm PE-50 tube was looped subcutaneously on the back of the rat to the surface of the neck. The catheters were passed subcutaneously to the posterior surface of the neck and placed in a plastic cup that was sutured to skin out of reach of the rat. The rats were placed in a CMA/120 system for freely moving animals after surgery, with free access to water and food, and given 24 h to recover.

Experimental Design. The microdialysis experiment started with a 2-h stabilization period. Ringer’s solution (R-CNS) containing 5 ng/ml D$_3$-morphine was perfused through the microdialysis probes by a CMA/100 microinjection pump with a flow rate of 1 µl/min (CMA Microdialysis). Samples were collected from the blood and brain probes at 20-min intervals throughout the experiment. Morphine was infused after the stabilization period using a syringe pump (Harvard Apparatus Inc., Holliston, MA). The infusion solutions contained 0.0326, 0.1627, and 0.8126 ng/ml total morphine in saline, whereas the concentration of $^{14}$C-morphine was kept constant in all the infusion solutions at 0.326 µg/ml.

The first bolus infusion containing $^{14}$C-morphine and unlabeled morphine in saline was administered for 10 min (Table 1), followed by a constant-rate infusion of the same solution over 110 min, to achieve a low steady-state plasma concentration. The rats subsequently received the second and third infusions in the same manner: 10-min bolus and 110-min constant-rate infusions to achieve medium (infused 120 min from the start of the study) and high (at 240 min) plasma concentrations ($n = 6$). Bolus doses and infusion rates were chosen to reach total steady-state plasma concentrations of 10 ng/ml, 50 ng/ml, and 250 ng/ml (Fig. 1). An additional rat received bolus and infusion doses to achieve ultralow (0.4 ng/ml), very low (2 ng/ml), and low (10 ng/ml) total steady-state concentrations (Table 1).

### Table 1

<table>
<thead>
<tr>
<th>Steady-State Level</th>
<th>Number of Rats</th>
<th>Bolus Infusion (10 min)</th>
<th>Constant Infusion (110 min)</th>
<th>Target Conc.</th>
<th>Measured Plasma Conc.</th>
<th>Measured Plasma Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$n$</td>
<td>$\text{ng} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$</td>
<td>$\text{ng} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$</td>
<td>$\text{ng} \cdot \text{ml}^{-1}$</td>
<td>$\text{ng} \cdot \text{ml}^{-1}$</td>
<td>$\text{ng} \cdot \text{ml}^{-1}$</td>
</tr>
<tr>
<td>Ultra Low low</td>
<td>1</td>
<td>0.0156</td>
<td>0.00156</td>
<td>0.4</td>
<td>0.16</td>
<td>6.93 ± 2.07</td>
</tr>
<tr>
<td>Very Low</td>
<td>1</td>
<td>0.0624</td>
<td>0.0078</td>
<td>2</td>
<td>1.12</td>
<td>41.2 ± 15.80</td>
</tr>
<tr>
<td>Low</td>
<td>7</td>
<td>0.4</td>
<td>0.04</td>
<td>10</td>
<td>8.5 ± 3.3</td>
<td>41.2 ± 15.80</td>
</tr>
<tr>
<td>Medium</td>
<td>6</td>
<td>1.56</td>
<td>0.20</td>
<td>50</td>
<td>47.2 ± 19</td>
<td>41.2 ± 15.80</td>
</tr>
<tr>
<td>High</td>
<td>6</td>
<td>7.80</td>
<td>0.98</td>
<td>250</td>
<td>208 ± 63</td>
<td>215 ± 60.5</td>
</tr>
</tbody>
</table>

* Each value is the mean of three measurements for each level for each rat.
The pharmacokinetic parameters used to calculate the infusion rates were obtained from Ekblom et al. (1993). Blood samples, taken at 45, 75, and 105 min during each infusion period, were collected in heparinized tubes. The plasma was harvested by centrifugation for 5 min at 10,000 rpm (7200g). The microdialysis and plasma samples were stored at −20°C until analysis. The rats were anesthetized after the experiment with isoflurane and euthanized by decapitation. The brains were stored at −20°C until analysis.

**Drug Analysis.** Because of the large number of samples from microdialysis, low-concentration samples were analyzed by AMS and high-concentration samples were analyzed by LC-MS/MS. Medium-concentration samples were analyzed by both AMS and LC-MS/MS. For rat 7 (very low concentrations), all samples were analyzed by AMS. The samples were purified by liquid chromatography (LC) to extract metabolites of morphine before analysis.

**Sample Purification for AMS.** The LC system used to remove the morphine metabolites for AMS analysis consisted of two pumps (Shimadzu LC-10AD; Shimadzu, Kyoto, Japan), a Hynpurity C18 column (10 × 4 mm, 3-μm particle size; Dalclo Chromtech, Hagersten, Sweden), and a triathlon autosampler (Spark Holland, Emmen, The Netherlands) equipped with a 200-μl loop, a 100-μl syringe, and an extra six-port valve to enable column switching.

For the microdialysis samples, 10 μl was injected directly. A mobile phase consisting of 2% methanol in H2O was used to elute morphine-3-glucuronide to waste over 3 min. After 3 min, the valve was switched and a mobile phase consisting of 70% ACN and 30% 5 mM ammonium acetate was used to elute the morphine fraction, which was collected over 2 min. Both pumps were operated with a flow rate of 0.5 ml/min. The collected fractions were analyzed by AMS.

For the plasma samples, 50 μl was precipitated with 100 μl of ACN containing 50 ng/ml D3-morphine as internal standard. The sample was vortexed and centrifuged, and 50 μl of the supernatant was evaporated under N2 at 45°C. The residue was dissolved in 250 μl of MilliQ water, vortexed and centrifuged, placed in an ultrasonic bath for 10 min, and vortexed and centrifuged again. Ten microliters was injected into the LC system as described above for the microdialysis samples. The collected fractions were analyzed by AMS.

**Accelerator Mass Spectrometry.** The experimental method for AMS, used in this study to determine 14C-morphine concentrations in plasma and microdialysis samples from blood and brain ISF in low- and medium-concentration ranges, has been described previously (Salehpour et al., 2008a,b, 2009) and will be outlined only briefly. The Uppsala 5 MV Pelletron Tandem Accelerator (NEC, Middleton, WI) was used to measure the 14C/12C isotopic ratio and, thus, the concentration of morphine.

The principles of the sample preparation method were described previously by Salehpour et al. (2008a). The collected samples (plasma, blood microdialysis, brain microdialysis, and blanks) underwent LC separation as described above. The fraction corresponding to unbound morphine was collected and freeze-dried (Scanvac Modulespin 40; Scanlab A/S, Denmark) for approximately 4 h at 2000 rpm under a vacuum of approximately 10−3 mbar. Tributyrin (C15H26O6, 1.5 mg), a 14C-depleted carbon carrier compound, was added to the dried sample and placed in a quartz tube with 80 mg of CuO as the oxidizing agent. The tube was evacuated to a pressure of approximately 10−3 mbar, flame-sealed, and subsequently baked at 950°C for 3 h to produce CO2 gas. The gas was cryogenically purified and transferred in vacuum to a vial containing 80 mg of zinc powder as the reducing agent and 2 mg of iron powder as the catalyst. The sealed vial containing the CO2 gas was baked at 530°C for 6 h, and the resultant graphite (approximately 1 mg) was pressed and loaded into the ion source of the accelerator.

Samples were analyzed for 5 min each, after which a reference sample (oxalic acid II; NIST, Boulder, CO) was measured. The process was repeated two or three times, depending on the count rate from the 14C detector. The fractionation effects were corrected by measuring the 14C/12C ratio of a small part of the CO2 gas, using an off-line isotope ratio mass spectrometer (652-Optima; Fisons/ VG-Isotopic, Manchester, UK). The fractionation-corrected isotopic ratios are presented in absolute modern 14C; the amount of [14C]morphine was determined as described previously by Salehpour et al. (2009). The morphine concentration in the samples was recalculated from the [14C]morphine concentrations obtained from AMS as they were mixed in fixed proportions.

**LC-MS/MS.** The experimental method for LC-MS/MS developed by Bengtsson et al. (2005), with minor adjustments, was used for the analysis of medium- and high-concentration plasma and microdialysis samples of morphine using electrospray ionization. The method was linear between 0.5 and 200 ng/ml for microdialysis samples with a coefficient of variation <13.8% and an accuracy of 95 to 104%. For sheep plasma, the method was linear between 2 and 2000 ng/ml with a coefficient of variation <6.0% and accuracy of 98 to 106% (Bengtsson et al., 2005). For the microdialysis samples, 5 μl of the microdialysate was injected directly into the system.

For the plasma samples, 50 μl was precipitated with 100 μl of ACN containing 50 ng/ml D3-morphine. After vortexing and centrifugation, 50 μl of the supernatant was evaporated under N2 at 45°C. The residue was dissolved in 200 μl of TFA (0.02%) and transferred to a polypropylene autosampler vial (kept at 10°C); 10 μl of the plasma sample was then injected into the system.

The chromatography system consisted of two pumps (Shimadzu LC-10AD), an injector (SIL-HT autosampler; Shimadzu, Kyoto, Japan), and a detector (Quattro Ultima triple quadrupole mass spectrometer; Micromass, Manchester, UK). MassLynx software (version 4.0; Waters, Sweden AB, Sollentuna, Sweden) was used for mass spectrometry control and spectral processing. A column switch system was used to remove salts from the samples. A Hynpurity C18 column (10 × 3 mm, 3-μm particle size; Dalclo Chromtech) was used for purification, and a ZIC HILIC column (50 × 4.6 mm, 5-μm particle size; SeQuant AB, Umeå, Sweden) was used for the analytical separation. The mobile phase consisted of 70% ACN and 30% 5 mM ammonium acetate. For the purification, 0.02% TFA was used. The flow rate for both pumps was 500 μl/min, and this rate was decreased to 260 μl/min before the sample entered the mass spectrometer using a flow split. The detector was set in positive ion mode. The parameters included cone gas (N2) at 280 l/h and desolvation gas (N2) at 1180 l/h. The source temperature was set at 130°C, and the desolvation temperature was set at 400°C. The capillary voltage was 3.00 kV, and the cone voltage was 75 V. The transitions were 285.9→151.9 m/z for morphine, 289.0→151.9 m/z for D3-morphine, and 462.2→285.9 m/z for morphine.
3-glucuronide. The brain samples were homogenized with a 5-fold volume (w/v) of perchloric acid (0.1 M) and then centrifuged at 1500 g for 10 min. A slightly modified version of the solid-phase extraction method by Joel et al. (1988) was used to pretreat 100 µl of the supernatant and 50 µl of internal standards at a concentration of 25 ng/ml. Methanol (3 ml) was used for elution, and the eluate was evaporated at 45°C under nitrogen. The residue was redissolved in 200 µl of TFA (0.02%), and 10 µl was injected into the system as described above. The standards were 0.2 to 256 ng/ml for microdialysis samples, 1 to 500 ng/ml for plasma, and 2 to 500 ng/g for brain.

Calculations. The recovery of morphine for each probe during each sample interval was calculated using the calibrator method (Bengtsson et al., 2008), with D3-morphine in the perfusate (eq. 1)

\[
\text{Recovery} = \frac{C_{\text{in}} - C_{\text{out}}}{C_{\text{in}}} \quad (1)
\]

where \(C_{\text{in}}\) is the concentration of D3-morphine in the perfusate and \(C_{\text{out}}\) is the concentration of D3-morphine in the dialysate. The average recovery for the whole experiment was used for each probe, because the recovery of morphine did not change with time.

The concentration of unbound morphine in the blood and brain ISF was calculated as

\[
C_{u,\text{brain,ISF}} = \frac{C_{\text{dialysate}}}{\text{Recovery}} \quad (2)
\]

The partition coefficient for the unbound morphine concentration \(K_{p,uu}\) was calculated as

\[
K_{p,uu} = \frac{C_{u,\text{brain,ISF}}}{C_{u,\text{blood,ISF}}} \quad (3)
\]

\(K_{p,uu}\) was determined for each rat at each concentration using the last three samples from the blood and brain probes.

The volume of distribution of unbound drug in the brain, \(V_{u,\text{brain}}\), was calculated as

\[
V_{u,\text{brain}} = \frac{A_{\text{tot,brain}} - V_{\text{blood}} \times C_{\text{tot,blood}}}{C_{u,\text{brain,ISF}}} \quad (4)
\]

where \(A_{\text{tot,brain}}\) is the concentration of morphine (ng/g) in each total brain sample, \(V_{\text{blood}}\) is the concentration of morphine in the last blood sample at the highest concentration, and \(C_{u,\text{brain,ISF}}\) is the unbound morphine concentration in brain in the last microdialysis sample. The parameter \(V_{\text{blood}}\) was set to 14 µl/g (Hammarlund-Udenaes et al., 2008).

Student’s t test was applied to compare the \(K_{p,uu}\), \(C_{u,\text{brain}}\), and \(C_{u,\text{blood}}\) values from LC-MS/MS and AMS. Analysis of variance with one factor was applied to compare the \(K_{p,uu}\) values for the rat 7 (very low concentrations).

Results

The total plasma concentrations of morphine measured by LC-MS/MS (Fig. 2) showed rapid attainment of steady state using this infusion scheme. Steady state had been reached at the time of the first plasma sample, 45 min after the start of the bolus infusion at each concentration level. One plasma sample for each level from each rat...
was also analyzed by AMS. There were no significant differences found between the concentrations measured by the two analytical methods (Table 1; Fig. 2) \((p = 0.1)\). Furthermore, the measured plasma concentration values showed good agreement with the theoretically predicted concentrations.

The average recovery of morphine was \(62 \pm 10\%\) for the blood probe and \(12 \pm 4\%\) for the brain probe. The unbound morphine concentration in the brain remained lower than that in the blood for all infusion rates (Fig. 3). With AMS, the average \(K_{puu}\) values for the low and medium steady-state levels were \(0.22 \pm 0.08\) and \(0.21 \pm 0.05\), respectively (not significant; \(p = 0.5\)). With LC-MS/MS, \(K_{puu}\) values for the medium and high steady-state levels were \(0.24 \pm 0.05\) and \(0.26 \pm 0.05\), respectively (not significant; \(p = 0.2\)). The additional rat with even lower steady-state levels (Table 1) was studied because the \(K_{puu}\) values for low, medium, and high steady-state levels of morphine were essentially the same. \(K_{puu}\) values were \(0.16 \pm 0.01\), \(0.16 \pm 0.02\), and \(0.18 \pm 0.03\) for the low, very low, and ultralow plasma concentrations, respectively, when measured by AMS. The difference between these values also failed to reach statistical significance \((p = 0.6)\). Thus, there were no significant changes in \(K_{puu}\) over a 625-fold concentration range \((0.4–250\,\text{ng/ml})\) (Fig. 4).

The medium steady-state level was used to compare the absolute concentration values obtained using LC-MS/MS and AMS. Although the values measured by the two methods for the plasma samples were indistinguishable, significant differences were found for the microdialysis samples. AMS resulted in 38% higher concentration values than LC-MS/MS for the blood microdialysis samples \((p = 0.000024)\) and 13% higher concentrations for the brain microdialysis samples \((p = 0.06)\) (Fig. 3). These differences were also evident when the ratio of AMS to LC-MS/MS was plotted against the corresponding LC-MS/MS values to show the accuracy of AMS when considering LC-MS/MS as the standard (Fig. 5). As a result, the \(K_{puu}\) values were, on average, 16% lower when the samples were analyzed with AMS than with LC-MS/MS. Although these variations do not affect our conclusions in these experiments, they should be investigated for future studies that might require absolute comparisons.

The average total brain concentration measured by LC-MS/MS was \(70.2 \pm 9.5\,\text{ng/g brain}\), giving an average volume of distribution for unbound morphine in the brain of \(2.6 \pm 0.7\,\text{ml/g brain}\) (eq. 4).

**Discussion**

The objective of this study was to investigate the BBB transport of morphine over a wide concentration range, including low concentrations, using AMS and LC-MS/MS for the analysis of microdialysis samples. In general, microdialysis samples require highly sensitive analytical methods because of the small volume and the very low concentrations of the dialysate; the dialysate concentrations are only a fraction of the surrounding in vivo unbound drug concentrations. In our

**Fig. 4.** Average ± S.D. of \(K_{puu}\) values for individual rats plotted against the average steady-state morphine concentration in plasma. Open symbols represent the \(K_{puu}\) analyzed by LC-MS/MS; closed symbols represent the \(K_{puu}\) analyzed by AMS. \(C_{ss}\), steady-state plasma concentrations.

**Fig. 5.** AMS to LC-MS/MS ratio for the morphine plasma concentration (diamonds) and unbound blood (squares) and brain (triangles) concentrations plotted against the corresponding LC-MS/MS values.
study, that fraction was 12% for microdialysis samples from the brain. It was apparent from this study that a higher sensitivity than what is possible with LC-MS/MS was needed. AMS meets the required criteria perfectly and provides a means of studying these very low concentrations.

Active influx at the BBB is a rare characteristic known for very few drugs; it not only affects the pharmacokinetics of the drugs in the brain, but it also presents new opportunities for development of drugs with the brain as target organ. Although no active uptake transporter has yet been characterized for morphine, we do know that at least one is present at the BBB in sheep (Bengtsson et al., 2009). In addition, oxycodone, being a very similar compound to morphine, showed a 3-fold higher unbound concentration in the brain than blood in rats. This result confirms the presence of an active uptake transport system for opioids, making it very likely that there is also an active uptake of morphine in rats. Because the active uptake transporter has not yet been characterized (Okura et al., 2008), the uptake process could not be studied by blocking it with a specific blocker, and saturating efflux transport is not achievable in vivo. There has consequently been conjecture that it might be possible to separate the influx and efflux processes in vivo by decreasing the concentrations of morphine (Xie et al., 1999; Groenendaal et al., 2007).

In this study, unbound morphine concentrations in the brain remained significantly lower than the unbound morphine concentrations in the blood within the 625-fold concentration range studied. There was no change in the $K_{p,un}$ of morphine within the plasma concentration range of 0.4 to 250 ng/ml. These results do not confirm the indications given in the study by Xie et al. (1999), which show that active influx of morphine can be observed at very low concentrations. The discrepancy between the studies might be due to species differences in the experimental models used. However, although the inclusion of active influx increased the fit of the model proposed by Groenendaal et al. (2007), there was no concrete evidence suggesting the presence of such a process in their study. In our experimental setup, we used a 25-fold lower concentration than the $K_{50}$ described by Groenendaal et al. (2007), but no active influx of morphine was seen. This result does not necessarily mean that there is no active influx of morphine at the BBB, but rather that the evidence indicated that it could not, based on concentration, be differentiated from the more efficient efflux.

The use of AMS, in combination with advanced sampling techniques such as microdialysis for pharmacokinetic studies, is a very useful and elegant approach. Our study uses the sensitivity of AMS to study the behavior of morphine at a very low concentration at the BBB using microdialysis and comparing AMS with LC-MS/MS. Other researchers have recently developed a method of combining AMS and microdialysis for studying the BBB transport of plant polyphenols (Janle et al., 2010).

The $K_{p,un}$ values in our study were, on average, 16% lower with AMS than with LC-MS/MS, which may have been due to the different approaches of the methods and the multiple clean-up and metabolite separation steps. Another study comparing the two methods for analysis of biological samples yielded similar deviations, with 20% lower values for AMS than for LC-MS/MS (Miyaji et al., 2009). It is suggested that standards of known concentration should be included with the samples during analysis with AMS to provide a better picture of the method’s accuracy.

In conclusion, decreasing the concentration of morphine did not affect the $K_{p,un}$, showing that it was not possible to separate the active uptake transport process from active efflux using this method.

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Authorship Contributions

Participated in research design: Sadiq, Salehpour, Forsgard, Possnert, and Hammarlund-Udenaes.

Conducted experiments: Sadiq, Salehpour, Forsgard, and Possnert.

Contributed new reagents or analytic tools: Sadiq and Salehpour.

Performed data analysis: Sadiq, Salehpour, and Hammarlund-Udenaes.

Wrote or contributed to the writing of the manuscript: Sadiq, Salehpour, Forsgard, and Hammarlund-Udenaes.

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