Morphine brain pharmacokinetics at very low concentrations studied with Accelerator Mass Spectrometry and Liquid Chromatography-tandem Mass Spectrometry

Muhammad Waqas Sadiq, Mehran Salehpour, Niklas Forsgard, Göran Possnert, Margareta Hammarlund-Udenaes

Department of Pharmaceutical Biosciences, Uppsala University, Box 591, SE-75124 Uppsala, Sweden (M.W.S., M. H-U)

Department of Physics and Astronomy, Ion Physics, Ångstrom Lab., Uppsala University, Box 534, SE-751 21 Uppsala, Sweden (M.S., N.F., G.P.)
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B. Corresponding author:

Margareta Hammarlund-Udenaes

Division of Pharmacokinetics and Drug Therapy

Department of Pharmaceutical Biosciences

Uppsala University

Box 591

SE-75124 Uppsala

Sweden

Tel: +46 18 4714300

Fax: +46 18 4714003

Email: mhu@farmbio.uu.se

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D. Abbreviations Used

BBB – blood-brain barrier

AMS – accelerator mass spectrometry

LC-MS/MS – liquid chromatography-tandem mass spectrometry

$C_{\text{ss}}$ – plasma steady-state concentration

CNS – central nervous system

ISF – interstitial fluid

$C_{u,\text{tissue}}$ – unbound drug concentration of tissue

$K_{p,\text{uu}}$ – partition coefficient for unbound drug

$V_{u,\text{brain}}$ – Volume of distribution of unbound drug in brain

NS – not significant
ABSTRACT

Morphine has been predicted to show nonlinear blood-brain barrier (BBB) transport at lower concentrations. Present study investigated the possibility of separating active influx of morphine from its efflux by using very low morphine concentrations, and to compare AMS with LC-MS/MS as method for analysing 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⁻¹), medium (50 ng.ml⁻¹) and low (10 ng.ml⁻¹) steady-state plasma concentrations (Css). An additional rat received infusions to achieve low (10 ng.ml⁻¹), very low (2 ng.ml⁻¹) and ultra low (0.4 ng.ml⁻¹) concentrations. Unbound morphine concentrations from brain extracellular fluid and blood were sampled with microdialysis and analysed by LC-MS/MS and AMS. The average $K_{p,uu}$ for the low and medium steady-state levels were 0.22±0.08 and 0.21±0.05, when measured with AMS (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, measured with LC-MS/MS (NS; p=0.2). For the low, very low and ultra low levels, $K_{p,uu}$ values were 0.16±0.01, 0.16±0.02 and 0.18±0.03, respectively, measured with AMS. The medium-concentration $K_{p,uu}$ values were, on average, 16% lower with AMS than with LC-MS/MS. There were no significant changes in $K_{p,uu}$ over a 625-fold concentration range (0.4-250 ng.ml⁻¹). It was not possible to separate active uptake transport from active efflux using these low concentrations. The two analytical methods provided indistinguishable results for blood plasma concentrations, but differed by up to 38% for microdialysis samples; however, this did not affect our conclusions.
INTRODUCTION

In order to enter the CNS via the blood system, a drug must pass the blood–brain barrier (BBB), which consists of endothelial cells connected by tight junctions. The exposure to the brain of unbound drug is determined by the chemical structure of the drug and by interactions between the drug and local transporters and membranes (Friden et al., 2009). Any of 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 moderate to severe pain, acts on the central nervous system. This drug is a substrate for efflux transporters in rats and humans; studies have indicated that the ratio of unbound morphine concentration in the brain interstitial fluid (ISF) to that in blood ($K_{pu,u}$) is 0.29 in rats, 0.47 in pigs and 0.64 in humans with brain trauma, indicating net active efflux from the brain (Xie et al., 1999; Tunblad et al., 2003; Ederoth et al., 2004; Tunblad et al., 2004). In one study, co-administration of probenecid decreased the brain efflux of morphine, thus increasing the $K_{pu,u}$ from 0.29 to 0.39 (Tunblad et al., 2003). This indicates that morphine is a substrate for nonspecific probenecid-sensitive transporters. Oral pretreatment of rats with the specific P-glycoprotein (Pgp)-inhibitor GF120918 caused a prolonged morphine-associated antinociceptive effect, due to morphine's prolonged half life in the brain (Letent et al., 1999). The involvement of Pgp in the efflux of morphine at the BBB is also supported by the results of modeling to describe the transport of morphine, as proposed by Groenendaal et al., where the efflux rate constant values decreased from 0.0195 to 0.0113 (min$^{-1}$), to provide the best fit after addition of Pgp-inhibitor GF120918 (Groenendaal et al., 2007). In a study of Pgp-deficient mice, the influx clearance ($CL_{in}$) for morphine was 24% higher than that for wild-
type mice, which indicates that Pgp only accounts for part of the efflux from the BBB (Dagenais et al., 2004).

BBB transport of morphine in sheep differs from that in other species. There is clear active influx of morphine, with a $K_{p,uu}$ 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 oxycodone in the rat brain than in blood (i.e. $K_{p,uu} = 3$) (Bostrom et al., 2006). This is in sharp contrast to the net active efflux of morphine across the BBB, even though morphine and oxycodone have similar structures and similar lipophilic properties (Bostrom et al., 2006). There are also studies indicating that morphine is actively transported into the brain in some species. Xie and coworkers found that, in mice, there was a tendency towards a higher $K_{p,uu}$ at low plasma morphine concentrations (Xie et al., 1999). This 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 oxycodone 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 knock-out 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 the analysis of such low concentrations was addressed by using AMS in combination with Liquid Chromatography with tandem Mass Spectrometry (LC-MS/MS). A second aim of the 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, Inc. (St. Louis U.S.A.). Morphine hydrochloride ampoules (Morfin Meda 10 mg/mL) were obtained from Apoteket (Uppsala, Sweden). Isoflurane Baxter was purchased from Baxter Medical AB, Kista, Sweden. Ammonium acetate, acetonitrile (ACN) and trifluoroacetic acid (TFA) were purchased from Merk, Darmstadt, Germany. D3-morphine hydrochloride was purchased from Lipomed, Arleshem, Switzerland. The Ringers 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, Bedford, MA). Physiological saline was purchased from Braun (Braun Medical AB, Stockholm, Sweden).

Animals

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

Animal Surgery

The rats ($n=7$) were anaesthetised with Isofluran Baxter (2.5%) balanced with oxygen (1.5 L.min$^{-1}$) and nitrous oxide (1.5 L.min$^{-1}$). 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⁻¹ (Heparin Leo1, 5000 IE.mL⁻¹, Leo Pharma AB, Malmö, Sweden). A CMA/20 (10mm) probe (CMA Microdialysis) was inserted into the right jugular vein through a guide cannula and was fixed with two stitches to the pectoral muscle. The rats were 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 (Dentalon1 Plus, Heraeus, Hanau, Germany). A CMA/12 (3mm) probe was inserted after fixing the guide cannula. In order 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 sutured to the 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 were given 24 h to recover.

**Experimental Design**

The microdialysis experiment started with a two-hour stabilisation period. Ringer solution (R-CNS) containing 5 ng.ml⁻¹ deuterated (D₃-) morphine was perfused through the microdialysis probes by a CMA/100 microinjection pump with a flow rate of 1 µl.min⁻¹ (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, Massachusetts, USA). The infusion solutions contained 0.0326, 0.1627 and 0.8126 mg.mL⁻¹ total morphine in saline while the concentration of ¹⁴C-morphine was kept constant in all the infusion solutions at 0.326 µg.mL⁻¹.

The first bolus infusion containing ¹⁴C-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: a 10 min bolus and 110 min of
constant-rate infusion 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$^{-1}$, 50 ng.mL$^{-1}$ and 250 ng.mL$^{-1}$
(Fig. 1). An additional rat received bolus and infusion doses to achieve ultra low (0.4 ng.ml$^{-1}$),
very low (2 ng.ml$^{-1}$) and low (10 ng.ml$^{-1}$) total steady-state concentrations (Table 1).

The pharmacokinetic parameters used to calculate the infusion rates were obtained from
Ekblom et al. (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 minutes at 10000 rpm (7200g). The microdialysis and plasma samples
were stored at -20°C until analysis. The rats were anaesthetized 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
analysed by AMS and high-concentration samples were analysed by LC-MS/MS. Medium-
concentration samples were analysed by both AMS and LC-MS/MS. For rat 7 (very low
concentrations), all samples were analysed 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), a HyPurity C$_{18}$ column, 10 x 4 mm, 3 µm particle size (Dalco
Chromtech, Hägersten, Sweden), and a triathlon autosampler (Spark Holland, Emmen, The
Netherlands) equipped with a 100 µL loop, a 100 µL syringe and an extra six-port valve to enable column switching.

For the microdialysis samples, 10 µL were injected directly. A mobile phase consisting of 2% methanol in H₂O was used to elute M3G to waste over 3 minutes. After 3 minutes, 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 minutes. Both pumps were operated with a flow rate of 0.5 mL.min⁻¹. The collected fractions were analysed by AMS.

For the plasma samples, 50 µL was precipitated with 100 µL ACN containing 50 ng.mL⁻¹ 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 M.Q. water, vortexed and centrifuged, placed in an ultrasonic bath for 10 minutes, and vortexed and centrifuged again. Ten µl was injected into the LC system as described above for the microdialysis samples. The collected fractions were analysed by AMS.

**Accelerator Mass Spectrometry**

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

The principles of the sample preparation method are described in Salehpour et al. (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, Scanlaf A/S, Denmark) for about 4 hours at 2000 rpm under a vacuum of about $10^{-3}$ mbar. 1.5 mg of tributyrin ($C_{15}H_{26}O_6$), a $^{14}$C-depleted carbon carrier compound, was added to the dried sample and placed in a quartz tube with 80 mg CuO as the oxidizing agent. The tube was evacuated to a pressure of about $10^{-5}$ mbar, flame-sealed and subsequently baked at 950 °C for 3 hours to produce CO$_2$ gas. The gas was cryogenically purified and transferred in vacuum to a vial containing 80 mg zinc powder as the reducing agent and 2 mg iron powder as the catalyst. The sealed vial containing the CO$_2$ gas was baked at 530 °C for 6 hours and the resultant graphite (ca. 1 mg) was pressed and loaded into the ion source of the accelerator.

Samples were analyzed for 5 minutes each, after which a reference sample (Oxalic acid II, NIST, Bolder, Colorado, USA) was measured. The process was repeated 2-3 times, depending on the count rate from the $^{14}$C detector. The fractionation effects were corrected for by measuring the $^{13}$C: $^{12}$C ratio of a small part of the CO$_2$ gas, using an off-line isotope ratio mass spectrometer (Fisons/VG-Isotech 652-Optima, Manchester, UK). The fractionation-corrected isotopic ratios are presented in absolute Modern $^{14}$C; the amount of $^{14}$C-morphine was determined as described in Salehpour et al. (Salehpour et al., 2009). The morphine concentration in the samples was recalculated from the $^{14}$C-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., with minor adjustments, was used for the analysis of medium- and high-concentration plasma and microdialysis samples of morphine using electro-spray ionisation (Bengtsson et al., 2005). The method was linear between 0.5-200 ng.ml$^{-1}$ for microdialysis samples with CV < 13.8% and accuracy of 95 to 104%, for sheep plasma the method was linear between 2-2000 ng.ml$^{-1}$
with CV < 6.0% and accuracy 98-106% (Bengtsson et al., 2005). For the microdialysis samples, 5 µL of the microdialysate was directly injected into the system.

For the plasma samples, 50 µL was precipitated with 100 µL ACN containing 50 ng.mL⁻¹ 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 0.02 % TFA 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, Shimadzu Kyoto, Japan), an injector (SIL-HT Autosampler, Shimadzu Kyoto, Japan) and a detector – a triple quadrupole mass spectrometer (Quattro Ultima, Micromass, UK). MassLynx software (version 4.0) was used for MS control and spectral processing. A column switch system was used to remove salts from the samples. A HyPurity C₁₈ column, 10 x 3 mm, 3 µm particle size (Dalco Chromtech, Hägersten, Sweden), was used for purification and a ZIC HILIC column, 50 x 4.6 mm, 5µm particle size (SeQuant AB, Umeå, Sweden), was used for the analytical separation. The mobile phase consisted of 70% ACN and 30% 5 mmol.L⁻¹ ammonium acetate. For the purification, 0.02% TFA was used. The flow rate for both pumps was 500 µL.min⁻¹, decreased to 260 µL.min⁻¹ 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⁻¹ and desolvation gas (N₂) at 1180 L.h⁻¹. 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⁻¹ for morphine, 289.0→151.9 m.z⁻¹ for D₃-morphine, and 462.2→285.9 m.z⁻¹ for morphine 3-glucuronide. The brain samples were homogenised with five-fold volume (w/v) perchloric acid (0.1 mol.L⁻¹) and then centrifuged at 1500xg for 10 min. A slightly modified solid-phase extraction method by Joel et al. (Joel et al., 1988) was used to pretreat 100 µL of the
supernatant and 50 µL internal standards at a concentration of 25 ng.mL\(^{-1}\). Methanol (3 mL) was used for elution and the eluate was evaporated at 45°C under nitrogen. The residue was redissolved in 200 µL 0.02% TFA and 10 µL were injected onto the system as described above. The standards were 0.2 to 256 ng.ml\(^{-1}\) for microdialysis samples, 1 to 500 ng.ml\(^{-1}\) for plasma and 2 to 500 ng.g\(^{-1}\) 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.

\[
Recovery = \frac{C_{in} - C_{out}}{C_{in}} \tag{1}
\]

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

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

\[
C_{u,tissue} = \frac{C_{dialysate}}{Recovery} \tag{2}
\]

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

\[
K_{p,uu} = \frac{C_{u,brainss}}{C_{u,bloodss}} \tag{3}
\]

\(K_{p,uu}\) 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, \(V_{u,brain}\), was calculated as

\[
V_{u,brain} = A_{tot,brain} - V_{blood} \times C_{tot,blood} \times \frac{C_{u,brain ISF}}{C_{u,brain ISF}} \tag{4}
\]
Where $A_{\text{tot,brain}}$ was the concentration of morphine (ng g$^{-1}$) in each total brain sample, $C_{\text{tot,blood}}$ was the concentration of morphine in the last blood sample at the highest concentration and $C_{u,\text{brain ISF}}$ was the unbound morphine concentration in brain in the last microdialysis sample. The parameter $V_{\text{blood}}$ was set to 14 µL g$^{-1}$ (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. Anova with one factor was applied to compare the $K_{p,uu}$ values for the rat with 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 minutes after the start of the bolus infusion at each concentration level. One plasma sample for each level from each rat was also analysed 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 ± 10% for the blood probe and 12 ± 4% for the brain probe. The unbound morphine concentration in the brain remained lower than in the blood for all infusion rates (Fig. 3). With AMS, the average $K_{puu}$ for the low and medium steady-state levels was 0.22±0.08 and 0.21±0.05, respectively (NS; p=0.5). With LC-MS/MS, $K_{puu}$ for the medium and high steady-state levels was 0.24±0.05 and 0.26±0.05, respectively (NS; 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. $K_{puu}$ was 0.16±0.01, 0.16±0.02 and 0.18±0.03 for the low, very low and ultra low plasma concentrations, respectively, as 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 ng.ml$^{-1}$) (Figure 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). Consequently, the $K_{p,uu}$ 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±9.5 ng g$^{-1}$ brain, giving an average volume of distribution for unbound morphine in the brain of 2.6 ± 0.7 mL·g$^{-1}$ 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 surrounding in vivo unbound drug concentrations. In our study, that fraction was 12% for microdialysis samples from the brain. It was apparent from this study that 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. Not only does it affect 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). Also oxycodone, being a very similar compound to morphine, showed a 3-fold higher unbound concentration in the brain than blood in rats. This 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 our 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 $K_{p,uu}$ of morphine within the plasma concentration range 0.4 to 250 ng.ml$^{-1}$. These results do not confirm the indications given in the study by Xie et al. (Xie et al., 1999) 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. (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 $C_{50}$ described by Groenendaal et al. but no active influx of morphine was seen. This 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 like microdialysis for pharmacokinetic studies is a very useful and elegant approach. Our study utilizes the sensitivity of AMS in an effort to study the behavior of morphine at very low concentration at the BBB using microdialysis and comparing AMS with LC-MS/MS. Other workers have recently developed a method of combining AMS and microdialysis for studying the BBB transport of plant polyphenols (Janle et al., 2010).

The $K_{p,uu}$ 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 $K_{p_{uu}}$, 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, Hammarlund-Udenaes

Conducted Experiments: Sadiq, Salehpour, Forsgard, Possnert

Contributed new reagents or analytical tools: Sadiq, Salehpour

Performed data analysis: Sadiq, Salehpour, Hammarlund-Udenaes

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


Legends for figures

**Figure 1.** Study design for the microdialysis experiments, with a five-fold difference between each of the three morphine steady-state concentrations. Rapid attainment of the morphine steady-state levels was achieved by a combination of an initial 10 min bolus infusion followed by a 110 min constant-rate infusion (Table 1; low, very low and ultra low concentrations not plotted in this Figure.)

**Figure 2.** Total plasma concentrations of morphine in rats for all steady-state levels. Solid lines represent the aimed-for low, medium and high concentrations (n=6) while dashed lines indicate the aimed-for low, very low and ultra low morphine concentrations (n=1). Three samples were analysed by LC-MS/MS for each concentration level for each rat (empty symbols), while the last sample at each concentration level was also analysed by AMS (filled symbols).

**Figure 3.** Average±SD of unbound morphine concentrations in brain (diamonds) and blood (circles) for low, medium and high morphine steady-state levels as analysed by AMS (filled symbols) and LC-MS/MS (empty symbols) (n=6). The figure also shows the unbound morphine concentrations in brain (triangles) and blood (squares) for the ultra low, very low and low morphine steady-state levels analysed by AMS (n=1).

**Figure 4.** Average±SD of $K_{p,uu}$ values for individual rats plotted against the average steady-state morphine concentration in plasma. Empty symbols represent the $K_{p,uu}$ analysed by LC-MS/MS; filled symbols represent the $K_{p,uu}$ analysed by AMS.

**Figure 5.** AMS to LC-MS/MS ratio for morphine plasma concentration (diamonds), unbound blood (squares) and brain (triangles) concentration plotted against the corresponding LC-MS/MS values.
Table 1. Infusion scheme and resulting total plasma concentrations of morphine measured by LC-MS/MS and AMS. No significant differences were observed between the two methods when the same samples were compared.

<table>
<thead>
<tr>
<th>Steady-State Level</th>
<th>Number of rats (n)</th>
<th>Bolus Infusion (10min) (mg kg(^{-1}) h(^{-1}))</th>
<th>Constant Infusion (110min) (mg kg(^{-1}) h(^{-1}))</th>
<th>Target Conc. (ng ml(^{-1}))</th>
<th>Measured Plasma Conc. * LC-MS/MS (ng ml(^{-1}))</th>
<th>Measured Plasma Conc. AMS (ng ml(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultra Low</td>
<td>1</td>
<td>0.0156</td>
<td>0.00156</td>
<td>0.4</td>
<td>0.16</td>
<td>0.16</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>0.8</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>6.93±2.07</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.
Figure 5

- Plasma Conc.
- Cu Blood
- Cu Brain