JPP Journal of Pharmacy and Pharmacology

JPP 2008, 60: 171–178

Received August 15, 2007
Accepted November 6, 2007
DOI 10.1211/jpp.60.2.0005
ISSN 0022-3573

# Pharmacokinetics and brain uptake of AM-36, a novel neuroprotective agent, following intravenous administration to rats

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## Abstract

The plasma pharmacokinetics and brain uptake of the novel neuroprotective agent AM-36 (1-(2-(4-chlorophenyl)-2-hydroxy)ethyl-4-(3,5-bis-(1,1dimethylethyl)-4-hydroxyphenyl) methylpiperazine) were assessed over 72 h following i.v. administration to male Sprague–Dawley rats. At nominal i.v. doses of 0.2, 1 and 3 mg $kg^{-1}$, AM-36 exhibited an extremely large volume of distribution (18.2 – 24.6 L $kg^{-1}$) and a long terminal elimination half-life, ranging from 25.2 to 37.7 h. Over this dose range, AM-36 exhibited linear pharmacokinetics, with no apparent change in clearance, volume of distribution or dose-normalised area under the plasma concentration – time curve. AM-36 was very highly bound to plasma proteins (> 99.6%); however, this did not appear to affect the ability of AM-36 to permeate the blood–brain barrier. Following a single i.v. dose of AM-36 at 3 mg $kg^{-1}$ to rats, brain concentrations were detected for up to 72 h, and the brain-to-plasma ratios were high at all time points (ranging from 8.2 at 5 min post-dose to 0.9 at 72 h post-dose). The very high brain uptake of AM-36 supports previous in-vivo efficacy studies demonstrating the neuroprotective effects of this compound when administered to rats with middle cerebral artery occlusion.

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**Acknowledgements:** The authors gratefully acknowledge Zenyth Therapeutics Ltd, Melbourne, Australia (formerly AMRAD Corporation Ltd) for their contribution to the discovery of AM-36, and Dr Alan Robertson, formerly of AMRAD, and Dr David Shackleford and Dr Alison Gregg (Centre for Drug Candidate Optimisation) for their contributions to the project.

## Introduction

There is increasing interest in the development of drugs with multiple or hybrid pharmacological actions that provide a synergistic beneficial response for the treatment of a variety of neurological disorders. Such hybrid drugs can act on a multitude of pathological processes triggered by neurological disorders such as stroke, Huntington's disease, Alzheimer's disease and Parkinson's disease (Jarrott et al 1999; de Lago et al 2005; Youdim & Buccafusco 2005). While it is theoretically feasible to administer a combination of different compounds with individual pharmacological actions to act on each of the pathological events, such compounds may exhibit quite significant differences in oral bioavailability, tissue distribution and clearance characteristics, which may result in a complicated dosage regimen and a consequent reduction in patient compliance. Such issues are avoided if a single compound with hybrid or multiple pharmacological actions is administered. In order to use such compounds for the treatment of neurological disorders, it is essential to characterise their pharmacokinetic properties and, in particular, their ability to permeate the blood–brain barrier (BBB) – the endothelial lining separating the blood from the brain compartment – to ensure that concentrations are sufficient to achieve the desired pharmacological effects.

Recent in-vitro and in-vivo studies in our laboratory have demonstrated that 1-(2-(4-chlorophenyl)-2-hydroxy)ethyl-4-(3,5-bis-(1,1dimethylethyl)-4-hydroxyphenyl) methylpiperazine (AM-36; Figure 1) is a hybrid compound consisting of an antioxidant moiety (3,5-terbutyl-4-hydroxytoluene) linked to a 4-arylalkyl piperazine group, which confers $Na^+$-channel blocking properties as well as sigma receptor antagonism to the molecule (Papanikos et al 2002; Callaway et al 2003, 2004). AM-36 has been shown to have

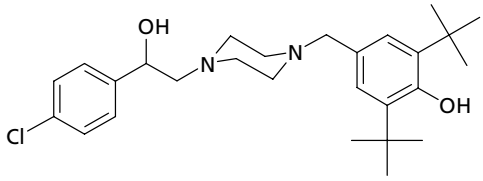


**Figure 1** Structure of the novel neuroprotective agent AM-36 (1-(2-(4-chlorophenyl)-2-hydroxy)ethyl-4-(3,5-bis-(1,1dimethylethyl)-4-hydroxyphenyl) methylpiperazine).

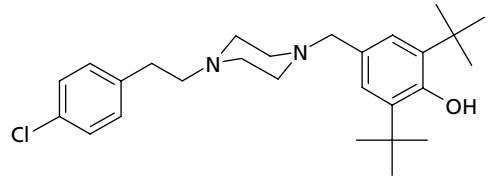


**Figure 2** Structure of AM-92, the internal standard used in the HPLC analysis of AM-36.

antioxidant properties (Jarrott et al 1999) and is also capable of inhibiting neuronal apoptosis (Callaway et al 2001). AM-36 is neuroprotective when administered up to 3 h after endothelin-1 induced middle cerebral artery occlusion, reducing both cortical and striatal damage (Callaway et al 1999). In addition to improving functional deficits in this stroke model, more recently AM-36 has been shown to directly modulate the neutrophil inflammatory response and to reduce BBB breakdown following an ischaemic attack (Weston et al 2006).

While there has been extensive pharmacological assessment of this novel compound for the treatment of stroke, there have been no reports on the plasma pharmacokinetics of this compound following administration to animals used in preclinical drug development. Given the neuroprotective effects of AM-36, the brain disposition and exposure of this compound following systemic administration also need to be fully understood. The aim of the current study was to assess the plasma pharmacokinetics of AM-36 following i.v. administration to rats at various doses, and to assess the brain uptake of AM-36.

## Materials and Methods

### Materials

AM-36 was synthesised by AMRAD Operations Pty Ltd (Melbourne, Australia) and isolated as the ditartrate salt. AM-92 (used as the internal standard (IS) for HPLC; Figure 2) was also synthesised by AMRAD Operations Pty Ltd. Diazepam (used as an IS for liquid chromatography–mass spectrometry (LCMS)) was purchased from Sigma Aldrich (St Louis, MO, USA). [$^{14}$C]-Inulin was obtained from American Radiolabeled Chemicals, Inc. (St Louis, MO, USA), [$^{3}$H]-diazepam, [$^{3}$H]-verapamil, [$^{14}$C]-mannitol, Solvable and Ultima Gold were purchased from Perkin Elmer (Boston, MA, USA); 30% v/v hydrogen peroxide was purchased from Ajax Finechem (New South Wales, Australia). Acetonitrile, methanol, DMSO, formic acid and all other solvents were of HPLC or analytical grade. Water was obtained from a Milli-Q water purification system (Millipore, Milford, MA, USA).

### Surgical procedures

All animal studies were performed in accordance with the Australian and New Zealand Council for the Care of Animals in Research and Training Guidelines. The study protocol was approved by the Monash University (Victorian College of Pharmacy) Animal Ethics Experimentation Committee. Male Sprague–Dawley rats (245–325 g) used for these studies were obtained from the Monash University animal breeding facility. Before experiments rats were housed in a 12 h light–dark cycle with free access to food and water.

Anaesthesia was induced using inhaled isofluorane (3%). A proprietary cannula (BASi Instruments, West LaFayette, IN, USA) was inserted into the right carotid artery and an indwelling polyethylene cannula was inserted into the internal jugular vein. The cannulae were sutured into position and exteriorised at the interscapular region. The animal was placed in a RaTurn animal housing system (BASi Instruments) and the carotid artery cannula was connected to a Culex automated blood sampler (BASi Instruments). Animals had free access to food and water during the post-surgery recovery period. Rats were then fasted overnight with free access to water. The same procedure was performed for brain uptake studies but a carotid cannula was not inserted, and rats were placed in standard individual animal cages for recovery and overnight fast.

### Pharmacokinetic studies

AM-36 ditartrate was administered by i.v. infusion of a 1 mL volume to rats via the indwelling jugular vein cannula over a 5 min period. The compound was administered at nominal free base equivalent doses of 0.2, 1 and 3 mg kg$^{-1}$, with three rats at each dose level. AM-36 was formulated in 0.9% v/v NaCl (for the 0.2 mg kg$^{-1}$ dose) or 5% w/v glucose (for the 1 and 3 mg kg$^{-1}$ dose; AM-36 has limited solubility in saline at higher concentrations).

Following commencement of the i.v. infusion, blood samples were obtained via the carotid artery cannula using a Culex autosampler. The autosampler was programmed to remove either 110 $\mu$L or 220 $\mu$L blood at 5, 10 and 25 min and 1, 2, 4, 8, 12, 18, 24, 36, 48 and 72 h post-dose. The total volume of blood taken over the entire 72 h experiment was less than 10% of the rat blood volume. Blood samples were collected directly into heparinised borosilicate tubes containing complete protease inhibitor cocktail (80 mg mL$^{-1}$, Roche, Mannheim, Germany), EDTA (0.1 M) and potassium fluoride (4 mg mL$^{-1}$), and were stored at 4°C in the autosampler. Following collection, blood samples were centrifuged, and the plasma supernatant was removed and stored at −20°C for subsequent analysis by LCMS.

### Brain uptake studies

AM-36 ditartrate was administered as a 200 $\mu$L bolus injection via the indwelling jugular vein cannula at a nominal dose of 3 mg kg$^{-1}$ (free base equivalent). To achieve this dose in this smaller volume, a more concentrated solution of AM-36 was required, and given the limited aqueous solubility of this compound, it was necessary to add 5% v/v DMSO to the aqueous formulation containing 5% w/v glucose. At 5 and 30 min and 2, 6, 10, 24, 48 and 72 h, rats were anaesthetised using inhaled isofluorane (3%) and 500 $\mu$L of blood was removed by cardiac puncture (n = 3 rats at each time point). Blood was collected into 1.5 mL Eppendorf tubes containing heparin, complete protease inhibitor cocktail (80 mg mL$^{-1}$), EDTA (0.1 M) and potassium fluoride (4 mg mL$^{-1}$). Samples were centrifuged immediately and the plasma supernatant collected. Immediately following blood collection, rats were humanely killed under anaesthesia, an incision was made through the back of the skull, and the brain, excluding the cerebellum, was removed and placed into preweighed polypropylene vials. Brain and plasma samples were stored at −20°C for subsequent analysis by LCMS.

### Plasma protein binding studies

The plasma protein binding of AM-36 was determined by spiking freshly obtained rat plasma with the compound at final concentrations of 1 and 2.5 $\mu$g mL$^{-1}$ (n = 2 at each concentration) and incubating at 37°C for 1 h. The solutions were then placed into plasma protein binding chambers (Amicon Inc., Beverly, MA) containing cellulose acetate filters (with a molecular weight cut-off of 30 000 Da) and centrifuged until approximately 60% of the total plasma volume had been filtered through the membrane. The concentrations of AM-36 in the filtrate and the unfiltered solution were determined by LCMS following extraction. To determine the degree of adsorption of AM-36 to the filter and chamber, phosphate-buffered saline (pH 7.4; PBS) solutions containing AM-36 at concentrations of 10 and 25 ng mL$^{-1}$ (representing 1% of the spiked plasma concentrations) were filtered in an identical fashion to the plasma samples. The concentration of AM-36 in the filtrate was then determined by LCMS following extraction.

### Partitioning studies

The whole-blood to plasma partitioning of AM-36 was determined by spiking freshly obtained rat blood with AM-36 at concentrations of 350, 450, 1500 and 2500 ng mL$^{-1}$ (n = 2 at each concentration). The blood was then incubated at 37°C for 1 h with gentle mixing, and divided into two portions. The first portion was centrifuged to obtain plasma, which was frozen for subsequent analysis of AM-36 by HPLC. The remaining blood was sonicated to induce lysis of red blood cells, centrifuged and assayed for AM-36 by HPLC.

### Physicochemical characterisation of AM-36

Physicochemical parameters were determined using the ACD log D suite of a commercial software program (version 9.0, Advanced Chemistry Development, Inc., Toronto, Canada). The physicochemical parameters included molecular weight, polar surface area, numbers of H-bond donors and acceptors, and number of freely rotating bonds.

The partitioning behaviour of AM-36 between octanol and aqueous buffer was determined as a measure of AM-36 lipophilicity. AM-36 was equilibrated at 25°C for 48 h in glass tubes containing 1 mL octanol and 1 mL PBS (67 mM phosphate). The tubes were inverted manually for 5 min and then at regular intervals over the equilibration period. After 48 h, the aqueous and organic phases were separated by centrifugation at 3000 rpm for 10 min, and an aliquot of each phase was withdrawn and viewed under a polarised light source to confirm the absence of particulate matter. The aliquots were diluted with acetonitrile:water (50:50) and assayed for AM-36 by HPLC. The distribution coefficient (D) between octanol and water was calculated by dividing the concentration of AM-36 in the octanol phase by the concentration in the aqueous phase.

### LCMS analysis

Solution, brain homogenate and plasma standards were freshly prepared for each analytical run, with each set of standards comprising at least eight different analyte concentrations. Diazepam was added as IS to 50 $\mu$L plasma to give a concentration of 0.4 $\mu$g mL$^{-1}$. Protein precipitation for plasma standards and samples was carried out by the addition of acetonitrile (120 $\mu$L), followed by vortexing and centrifugation. The supernatant was subsequently separated and 10 $\mu$L injected onto the column for LCMS analysis. Preweighed whole brains were homogenised in three volumes by weight of water containing EDTA (0.1 M) and potassium fluoride (4 mg mL$^{-1}$). Brain homogenate standards and samples were prepared by spiking brain homogenate (200 $\mu$L) with diazepam (0.1 $\mu$g mL$^{-1}$). Protein precipitation for brain homogenate standards and samples was carried out by the addition of acetonitrile (600 $\mu$L), followed by vortexing and centrifugation. The supernatant was subsequently separated and 10 $\mu$L injected onto the column for LCMS analysis.

Mass spectrometry was performed on a Micromass Quattro Premier triple quadrupole instrument coupled with a Waters Acquity Ultra Performance LC (Waters Corporation, Milford, MA, USA). Analytical separations were performed on a 50 × 1 mm, 4 $\mu$m Phenomonex polar $C_8(2)$ reverse-phase column equipped with a pre-column of the same material (Torrance, CA). Samples (10 $\mu$L) were injected onto the column and compounds were eluted using a binary gradient solvent system. Solvent A was Milli-Q water containing 2% v/v methanol and 0.05% v/v formic acid; solvent B was acetonitrile containing 0.05% v/v formic acid. AM-36 was analysed using the following gradient profile: 0–0.3 min, 2% B; 0.3–0.4 min, 2–20% B; 0.4–2.7 min, 20–80% B; 2.7–2.8 min, 80–95% B; 2.8–3.5 min, 95% B; 3.5–4.5 min, 95–2% B, followed by 2 min equilibration at the initial conditions. The peak of interest eluted at 2.7 min at a flow rate of 0.15 mL min$^{-1}$. Mass spectrometry was performed with positive-mode electrospray ionisation with a capillary voltage of 3.3 kV and source block and desolvation temperatures of 90°C and 350°C, respectively. Flow rates of the desolvation gas

(nitrogen) and collision gas (argon) were maintained at 500 L $h^{-1}$ and 0.38 mL $min^{-1}$, respectively. Inlet cone voltages were 30 and 40 eV for AM-36 and the IS, respectively, and collision energies of 30 and 27 eV were used. The elution of AM-36 and the IS were monitored using transitions of 459.19 → 219.27 and 285.17 → 154.02, respectively. Data acquisition, peak integration and quantitation were performed using Micromass Masslynx software (version 4.1, Waters Corp., Milford, MA, USA). Analyte concentrations were quantified by an external standard method using the system software (Quanlynx, Waters) with the calibration curve weighted to 1/x. The percentage deviation of the back-calculated concentrations relative to the nominal concentrations was determined after application of a second-order curve-fitting algorithm, and were 13.3% for plasma and 17.5% for brain at the lower limits of quantitation (0.5–1 ng $mL^{-1}$ and 5 ng $g^{-1}$, respectively).

### HPLC analysis

The concentration of AM-36 in plasma samples from blood–plasma partitioning studies and samples from octanol–water partitioning studies was determined by HPLC. To each 300 $\mu$L sample was added 50 $\mu$L of AM-92 as IS, 500 $\mu$L methanol and 1 mL sodium carbonate (2% v/v). This mixture was vortexed briefly, followed by the addition of 3 mL *tert*-butyl methyl ether. The sample was shaken vigorously for 5 min and centrifuged at 3000 rev $min^{-1}$ for 15 min at 20°C. The resulting supernatant was removed and transferred to a clean polypropylene tube. The extraction procedure was repeated with the remaining aqueous phase and the supernatant was removed and combined with the supernatant from the first extraction. The combined supernatants were evaporated under a gentle stream of nitrogen and then reconstituted in 500 $\mu$L acetonitrile:water (50:50) for injection onto the HPLC column.

Calibration standards were prepared by spiking blank rat plasma (or acetonitrile:water for samples obtained from octanol–water partitioning studies) with stock solutions of AM-36 and AM-92 (IS) prepared in acetonitrile:water; these samples underwent the same extraction procedure described above.

The HPLC system comprised a Beckman 126 solvent delivery system and a 168 UV detector (Beckman Coulter, Inc., Fullerton, CA, USA) with a detection wavelength of 214 nm. A 400 $\mu$L sample was injected onto a Beckman $C_{18}$ analytical column (4 mm × 25 cm, 5 $\mu$m particle size) at ambient temperature. To separate AM-36 and AM-92 from endogenous components, a two-component mobile phase was used. Phase A was 0.09% v/v trifluoroacetic acid in water; phase B was 0.1% v/v trifluoroacetic acid and 70% v/v acetonitrile in water. The mobile phase was delivered at a flow rate of 1 mL $min^{-1}$ using a linear gradient from 50 to 100% phase B over 15 min, maintained at 100% phase B for 10 min, and linearly decreased from 100% to 50% phase B over 5 min. The column was then re-equilibrated for 10 min before the next injection. The lower limit of quantitation using these conditions was 50 ng $mL^{-1}$, with a linearity range of 50 – 5000 ng $mL^{-1}$. Accuracy and precision for the plasma assay were determined by replicate injections of quality control samples. Satisfactory results were obtained for both precision (s.d. < 24% at 50 ng $mL^{-1}$ and < 5% at 5000 ng $mL^{-1}$) and accuracy (within ± 24% at 50 ng $mL^{-1}$ and ± 0.2% at 5000 ng $mL^{-1}$).

### Determination of brain microvascular plasma volume

[$^{14}$C]-Inulin was administered to rats to provide an estimate of the brain microvasculature plasma volume. Given its large molecular weight (5000 Da) and inability to permeate the BBB, any radioactivity present within brain homogenate following administration is assumed to be associated with the brain microvasculature. [$^{14}$C]-Inulin solution (200 $\mu$L 50 $\mu$Ci $mL^{-1}$ in normal saline) was administered to rats (n = 3), and brain and blood samples were collected 5 min later. A 50 $\mu$L aliquot of plasma supernatant was removed and placed into a 6 mL polyethylene scintillation vial. Ultima Gold scintillation cocktail (2 mL) was added to each of the vials, which were then briefly vortexed and analysed for radioactivity by liquid scintillation counting using a Tri-Carb 2800TR liquid scintillation counter (Perkin Elmer, Boston, MA, USA). The whole brain (with the cerebellum discarded) was placed into a preweighed 20 mL polyethylene scintillation vial and 4 mL solubilisation agent (Solvable) added to each vial, which were maintained at 50°C overnight to ensure total tissue solubilisation. Tissue samples were then bleached by addition of 600 $\mu$L hydrogen peroxide (30% v/v). Ultima Gold scintillation cocktail (15 mL) was added to each vial; samples were then briefly vortexed and analysed for radioactivity by liquid scintillation counting.

### Brain uptake of radiolabelled probe compounds

Brain uptake of radiolabelled probe compounds (mannitol, verapamil and diazepam) was determined in male Sprague–Dawley rats under conditions identical to those used for assessing the brain uptake of AM-36. Radiolabelled probe compounds were administered i.v. at a dose of 10 $\mu$Ci, and brain and plasma samples were obtained 5 min later and analysed by liquid scintillation counting (as described above).

### Data analysis

*Pharmacokinetic studies*

Pharmacokinetic parameters were estimated using the actual measured doses for each animal. Non-compartmental analysis was performed using WinNonlin software (version 4.0, Pharsight Corporation, Mountain View, CA, USA) to estimate the terminal elimination half-life, the total plasma clearance and the volume of distribution at steady state. All calculations were based on the area under the plasma concentration – time profile, calculated using the log-linear trapezoidal rule from time zero to infinity ($AUC_{0-\infty}$). The total blood clearance ($CL_b$) was calculated using the equation $CL_b = CL_{plasma}/R_{blood-plasma}$, where $CL_{plasma}$ is the plasma clearance and $R_{blood-plasma}$ is the whole-blood:plasma ratio of AM-36, which was calculated by dividing the concentration of AM-36 in the lysed red blood cell supernatant by the concentration of AM-36 in the plasma supernatant. The pharmacokinetic parameters obtained following i.v. administration of AM-36 to rats at different doses were compared

using a Kruskal–Wallis test with SPSS for Windows statistical package (version 15; SPSS, Chicago, IL, USA). All results are presented as mean ± s.d., unless stated otherwise.

*Brain uptake studies*

The brain uptake of AM-36 (or radiolabelled probe compound) was represented as a brain:plasma (B:P) ratio, according to the equation: $B:P = C_{brain}/C_{plasma}$ where $C_{brain}$ and $C_{plasma}$ are the concentrations of compound in the brain parenchyma homogenate (ng $g^{-1}$) and plasma (ng $mL^{-1}$), respectively, assuming equal density between brain homogenate and plasma (Garberg et al 2005). The concentration of compound detected in brain homogenate is a combination of that present in the brain parenchyma and that remaining within the cerebral vasculature. To correct for the concentration in the brain vasculature ($C_{brain\ vasculature}$), $C_{plasma}$ was multiplied by the plasma volume of the brain vasculature ($V_p$). $C_{brain}$ could then be determined by subtracting the concentration of compound detected in the brain vasculature ($C_{brain\ vasculature}$, in ng $g^{-1}$) from the concentration in the brain homogenate ($C_{brain\ homogenate}$).

In addition to calculating individual B:P ratios at discrete time points, the overall brain AUC of AM-36 was compared with the overall plasma AUC. Because of the destructive sampling technique required to obtain brain concentrations at each time point, Bailer's method was used to calculate the plasma AUC and brain AUC and the associated variances (Bailer 1988). To calculate the plasma AUC, it was necessary to obtain an estimate of the initial plasma concentration ($C_0$) following i.v. bolus dosing. Thus, non-compartmental analysis was applied to the mean AM-36 plasma concentration data using WinNonlin software to estimate $C_0$, and this value was used as the initial plasma concentration when calculating the plasma AUC using Bailer's method. The initial concentration of AM-36 in the brain was assumed to be zero. Statistical comparisons between the plasma AUC and brain AUC were then determined using the z-test.

*Plasma protein binding studies*

The percentage of AM-36 bound to plasma proteins was calculated from the equation: $\%\ bound = ([C_{plasma} - C_{filtrate}]/C_{plasma}) \times 100$, where $C_{plasma}$ is the concentration of AM-36 in unfiltered plasma (i.e. plasma containing proteins) and $C_{filtrate}$ is the concentration of AM-36 in the filtrate (i.e. following removal of plasma proteins).

## Results

### Pharmacokinetics of AM-36

Following i.v. administration of AM-36 to rats at nominal doses of 0.2, 1 and 3 mg $kg^{-1}$, plasma concentrations declined rapidly, with an extensive distribution phase and a relatively slow terminal elimination phase (Figure 3). The pharmacokinetic parameters obtained are shown in Table 1. Although not significantly different, the elimination half-life of AM-36 was longest at the lowest dose (37.7 ± 9.6 h), compared with values at 1 and 3 mg $kg^{-1}$ (26.6 ± 2.3 and 25.2 ± 4.6 h, respectively). The mean volume of distribution of AM-36 was extremely high and did not differ significantly over the dose range studied (18.2 – 24.6 L $kg^{-1}$), suggesting that AM-36 is distributed extensively into the peripheral tissue compartment. Using the Kruskal–Wallis test, no significant difference in the plasma clearance of AM-36 was observed over the dose range of 0.2 – 3 mg $kg^{-1}$. In order to calculate blood clearance from the i.v. plasma pharmacokinetic data, $R_{blood-plasma}$ of AM-36 was determined over the concentration range 320 – 2500 ng $mL^{-1}$, given that this represented the approximate in-vivo plasma concentrations observed following i.v. administration. The $R_{blood-plasma}$ of AM-36 ranged from 1.5 to 2.3, with a mean value of 1.85. Based on this value, the mean blood clearance of AM-36 ranged from 4.1 to 8.4 mL $min^{-1}$ $kg^{-1}$, with no statistical differences over the dose range studied. This linearity in the pharmacokinetics of AM-36 was further supported by the fact that the dose-normalised AUC was similar over the dose range of 0.2 – 3 mg $kg^{-1}$.

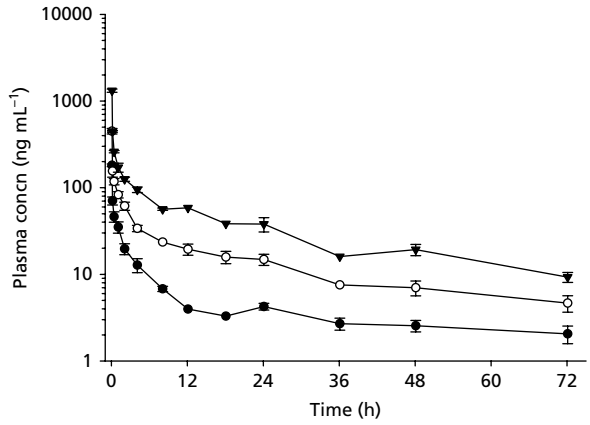


**Figure 3** Plasma concentrations of AM-36 following i.v. administration to male Sprague–Dawley rats at nominal doses of 0.2 (●), 1 (○) and 3 (▼) mg $kg^{-1}$. Data are mean ± s.e.m. (n = 3).

### Brain uptake of AM-36

The brain uptake of AM-36 following i.v. administration to rats at a nominal dose of 3 mg $kg^{-1}$ was monitored over 72 h; the resultant brain and plasma concentrations are shown in Figure 4. The brain concentrations of AM-36 were corrected for the amount of compound remaining in the brain microvasculature, through determining the plasma volume of the brain vasculature. This parameter was determined to be 26 ± 12 μL $g^{-1}$ brain tissue (n = 3), as assessed by the radioactivity in the brain following i.v. administration of [$^{14}$C]-inulin. This is similar to the vascular volume of rats determined by others (26 ± 10 μL $g^{-1}$) (Hsiao et al 2006).

The brain exposure (AUC) of AM-36 over 72 h, measured using Bailer's approach, was 7616.6 ± 1177.4 ng h $g^{-1}$, which was significantly different from the plasma AUC of 2561.0 ± 111.3 ng h $mL^{-1}$ ($P < 0.05$ using z-test). This resulted in an overall brain-to-plasma exposure ratio of 3.0, calculated by comparing brain AUC with plasma AUC. When measuring individual B:P ratios at discrete time points, AM-36 exhibited the greatest brain uptake at 5 min post-dose (with

**Table 1** Pharmacokinetic parameters obtained following i.v. administration of AM-36 to male Sprague–Dawley rats at nominal doses of 0.2, 1 and 3 mg $kg^{-1}$

| | Nominal dose (mg $kg^{-1}$) | | |
|---|---|---|---|
| | 0.2 | 1 | 3 |
| Measured dose (mg $kg^{-1}$) | 0.21 ± 0.01 | 1.01 ± 0.05 | 2.84 ± 0.06 |
| Terminal elimination half-life (h) | 37.7 ± 9.6 | 26.6 ± 2.3 | 25.2 ± 4.6 |
| Plasma clearance (mL $min^{-1}$ $kg^{-1}$) | 7.6 ± 2.7 | 13.7 ± 3.9 | 15.5 ± 2.1 |
| Blood clearance (mL $min^{-1}$ $kg^{-1}$)[a] | 4.1 ± 1.4 | 7.4 ± 2.1 | 8.4 ± 1.1 |
| Volume of distribution at steady state (L $kg^{-1}$) | 18.2 ± 3.3 | 23.5 ± 3.5 | 24.6 ± 3.9 |
| $AUC_{0-\infty}$/dose (h ng kg $mL^{-1}$ $mg^{-1}$) | 2351.4 ± 703.1 | 1273.6 ± 312.1 | 1091.7 ± 140.6 |

Data are mean ± s.d. (n = 3). [a]Calculated by dividing the plasma clearance by the blood-to-plasma ratio of 1.85.

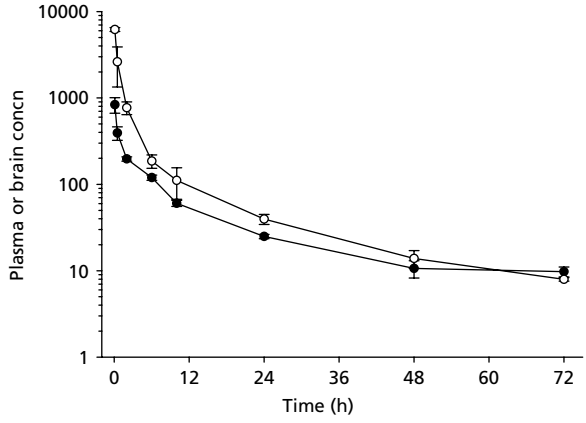


**Figure 4** Plasma (ng $mL^{-1}$; •) and brain (ng $g^{-1}$; ○) concentrations of AM-36 following i.v. administration of 3 mg $kg^{-1}$ to male Sprague–Dawley rats. Data are mean ± s.e.m. (n = 3).

a B:P ratio of 8.2), and this decreased in a time-dependent manner to 0.9 at the last time point. The B:P values of AM-36 at each time point are shown in Table 2. When comparing the brain uptake of AM-36 with that of other radiolabelled probe compounds (Figure 5), it is apparent that this neuroprotective agent has relatively high BBB permeability, even when compared with the CNS-active benzodiazepine, diazepam.

## AM-36 plasma protein binding

The binding of AM-36 to plasma proteins was investigated at plasma concentrations of 1 and 2.5 $\mu$g $mL^{-1}$, representing the upper concentration range of AM-36 measured in plasma following i.v. administration to rats. At each plasma concentration, AM-36 was not detected in the plasma filtrate by LCMS analysis, and so, using the lower limit of quantitation of the LCMS assay, the minimum value for plasma protein binding of AM-36 was calculated as 99.6%. Minimal adsorption of AM-36 to the filter and chamber was observed in control experiments conducted in PBS at concentrations of 10 and 25 ng $mL^{-1}$.

**Table 2** Brain-to-plasma (B:P) ratios of AM-36 following i.v. administration to male Sprague–Dawley rats at a nominal dose of 3 mg $kg^{-1}$

| Time (h) | B:P ratio |
|---|---|
| 0.08 | 8.2 ± 3.4 |
| 0.5 | 6.2 ± 5.4 |
| 2 | 3.9 ± 0.9 |
| 6 | 1.5 ± 0.4 |
| 10 | 1.8 ± 1.1 |
| 24 | 1.6 ± 0.3 |
| 48 | 1.3 ± 0.1 |
| 72 | 0.9 ± 0.3 |

Data are mean ± s.d. (n = 3).

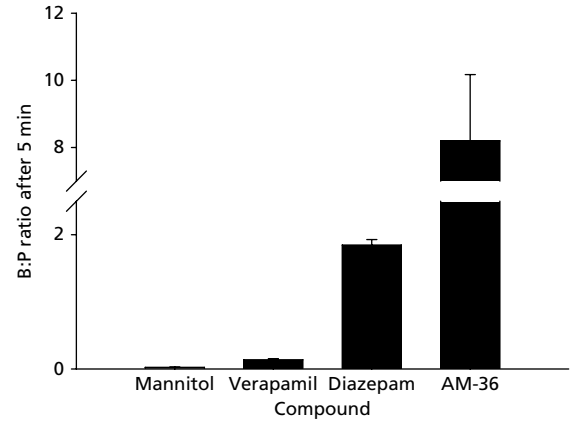


**Figure 5** Brain-to-plasma (B:P) ratio of radiolabelled probe compounds and AM-36 5 min after i.v. administration to male Sprague-Dawley rats. Data are mean ± s.e.m. (n = 3).

## Physicochemical properties of AM-36

The ACD-software-predicted and measured physicochemical properties of AM-36 are shown in Table 3. AM-36 has a molecular weight of 459.1 Da, a polar surface area of 46.9 $Å^2$,

**Table 3** Predicted and measured physicochemical parameters for AM-36, and recommended values for optimal BBB permeation

| Parameter | AM-36 value | Recommended value |
|---|---|---|
| Molecular weight (Da) | 459.1 | < 450[b] |
| Polar surface area (Å$^2$) | 46.9 | < 60[a] |
| No. H-bond donors | 2 | < 3[c] |
| No. H-bond acceptors | 4 | < 7[c] |
| No. freely rotating bonds | 9 | < 7[d] |
| Log $D_{octanol}$ | 4.5 | 1 – 3[b] |

[a]Kelder et al 1999; [b]van de Waterbeemd et al 1998; [c]Raub 2004; [d]Ajay et al 1999.

two H-bond donors, four H-bond acceptors and nine freely rotating bonds. In addition, the Log $D_{octanol}$ of AM-36 at pH 7.4 was measured experimentally to be 4.5. Table 3 includes values for the physicochemical properties often suggested for optimal BBB permeability.

## Discussion

In order to support the observed in-vivo neuropharmacological effects of AM-36 following administration to rodents (Callaway et al 1999; 2000; 2003; 2004; Weston et al 2006), the plasma pharmacokinetics and brain uptake of this compound were assessed following i.v. administration to rats. Across the dose-range of 0.2 – 3 mg kg$^{-1}$, AM-36 did not exhibit any evidence of non-linearity with each of the pharmacokinetic parameters measured, as assessed using the Kruskal–Wallis non-parametric test. AM-36 exhibited a very large plasma volume of distribution, which was most likely a result of the combined effect of high erythrocyte binding (as measured by a whole blood:plasma ratio of 1.85) and substantial partitioning into tissues. The large volume of distribution is consistent with the physicochemical properties of AM-36 (Table 3), which indicated a low degree of polarity and hydrogen bonding character and a high degree of lipophilicity. In addition, this large volume of distribution may be attributed to the high tissue binding, often observed with basic drugs that are ionised at physiological pH, which has been attributed to ion-pair interactions between the basic centre and the charged acidic head groups of phospholipid membranes (Smith et al 1996). The long elimination half-life of AM-36 would support dosing of this compound on a once-daily basis, a dosage regimen which has been demonstrated to protect against cortical and striatal neuronal damage in a rat model of stroke (Callaway et al 1999).

While monitoring of plasma concentrations is important in characterising the pharmacokinetics of a novel compound, the concentration of the drug at its site of action is of greater relevance. In this particular case, the concentration of AM-36 in the brain may provide insight into the duration of action of the compound. While AM-36 was highly bound to plasma proteins at concentrations representative of those detected in the plasma following i.v. administration, this appeared not to limit the ability of AM-36 to permeate the BBB, as shown by the high brain-to-plasma exposure ratio of approximately 3. The B:P ratios of AM-36 at discrete time points were initially very high (maximum mean value of 8.2) and decreased in a time-dependent manner to approximately 1 at 72 h. As shown in Figure 4, the brain concentrations of AM-36 appeared to decline more rapidly than the plasma concentration; however, there were still measurable concentrations of AM-36 in the brain 72 h after administration. Previous efficacy studies have demonstrated that AM-36 has neuroprotective effects for up to 72 h, although such studies were performed with a multiple dosing regimen (AM-36 being dosed every 24 h) (Callaway et al 1999). Given that the current study involved only a single dose of AM-36 for the assessment of BBB penetration, it would be anticipated that plasma and brain concentrations would be even higher with the multiple-dose regimen used in the efficacy studies. The long exposure of this compound in both the brain and plasma is considered to be an advantage for extended neuronal protection following an ischaemic attack.

The high brain uptake of AM-36 is not surprising given its favourable physicochemical properties (Table 3). As AM-36 has a very low polar surface area and a small number of H-bond donors and acceptors, passive permeation of this compound across the BBB is not expected to be restricted by physicochemical constraints (Kelder et al 1999; Raub 2004). While the number of freely rotating bonds of AM-36 exceeds the nominal recommended value for good BBB permeability (Table 3), it has been demonstrated that, of all physicochemical properties, including molecular weight, polar surface area and number of H-bond donors and acceptors, the number of freely rotatable bonds appears to have the least impact on BBB permeability (Ajay et al 1999; Garg & Verma 2006). The log D of AM-36 at pH 7.4 was also higher than the recommended range of 1 – 3 (van de Waterbeemd et al 1998), yet the brain permeation of this compound was very high, demonstrating the importance of not focusing unduly on one particular physicochemical property in determining the potential of a compound to permeate the BBB. The high brain uptake of AM-36 is also consistent with the observed neuroprotective effects following i.v. or intraperitoneal administration to rats with middle cerebral artery occlusion, where there was a significant reduction in cortical and striatal infarct volume and significant improvement in motor performance, neurological deficit and sensorimotor neglect tests (Callaway et al 1999, 2000, 2003, 2004; Weston et al 2006).

When the brain disposition of AM-36 5 min post-dose is compared with that of probe radiolabelled compounds, it is evident that AM-36 exhibits relatively high brain uptake. The B:P ratio of AM-36 5 min post-dose was approximately 3.3-fold higher than that of the CNS-active benzodiazepine, diazepam, which permeates the BBB by passive diffusion (Garberg et al 2005). Whether AM-36 permeates the BBB by passive diffusion or as a substrate for an active transport process present at the BBB is not known, and further studies would be required to provide further insight into these processes.

While the brain uptake of AM-36 was high in the healthy rats used in this study, it should be recognised that the disposition of compounds in the brain may vary in rats with

a BBB altered by ischaemic insult. Numerous reports have demonstrated altered brain uptake of compounds following ischaemic insult, with both increased uptake due to osmotic disruption and BBB breakdown (Anwar et al 1993; Albayrak et al 1997; Chi et al 2001) and decreased uptake due to disruption of sodium-dependent transporters (Stummer et al 1995; Kawai et al 1999). Whether AM-36 permeates the BBB solely by passive diffusion or is also a substrate for an active transporter process, it can be envisaged that the brain uptake of AM-36 may be altered by ischaemic insult. However, given the proven neuroprotective effects of this compound in diseased animals, any potential alteration in brain uptake of AM-36 does not appear to attenuate the pharmacological effects of this compound (Callaway et al 1999, 2000, 2003, 2004; Weston et al 2006).

## Conclusions

These studies have demonstrated that, following i.v. administration to rats, AM-36 exhibited an extremely long terminal elimination half-life and large volume of distribution. The clearance and dose-normalised AUC of AM-36 were not significantly different with doses ranging between 0.2 and 3 mg $kg^{-1}$, demonstrating that this compound exhibits linear pharmacokinetics over this dose range. AM-36 exhibited high brain uptake, and measurable concentrations were present in brain parenchyma for up to 72 h after a single i.v. dose of the compound, despite extensive plasma protein binding. The high brain uptake of AM-36 is consistent with previous studies demonstrating the neuroprotective effects of this compound following administration to rats with middle cerebral artery occlusion.

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