Journal of Neuroscience Methods 232 (2014) 199–206

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth

Basic Neuroscience

Brain penetration assessment *in vivo*: A reliable and simple method in anesthetized rats at steady state

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HIGHLIGHTS

- Brain penetration is measured in a robust and efficient manner at steady state.
- CNS drug discovery improved by simplified brain/plasma-ratio method in rats.
- Steady state method robust with respect to variable plasma–brain equilibration rate.
- One day experiment yielding brain/plasma-ratio in triplicates with simple equipment.
- The 3Rs benefits: animal reduction and steady state model refinement.

ARTICLE INFO

Article history:
Received 18 February 2014
Received in revised form 25 March 2014
Accepted 10 April 2014

Keywords:
Brain/plasma-ratio
In vivo
Steady state
Brain penetration
Drug discovery
Loading dose
Rat
3Rs-benefit

ABSTRACT

Background: For CNS drugs, brain disposition is of critical importance during drug discovery. *In vitro* methods are used early followed by more predictive *in vivo* methods later on in the drug discovery process. Current *in vivo* methods are costly, have long turnover times or do not measure brain disposition at steady state.
New method: A new method to evaluate drug brain disposition *in vivo* was developed in anaesthetized rats. Seven reference compounds were administered as an initial IV bolus (loading dose) followed by IV infusion for 4.5 h in order to obtain a steady state plasma concentration before brain sampling. The loading dose was estimated from a preliminary single dose IV pharmacokinetic study and was found to successfully bring plasma concentrations to steady state for compounds exhibiting either mono- or bi-compartmental pharmacokinetics.
Results: Using this method, a steady state lasting at least 2 h was obtained, thus making the *in vivo* method robust with respect to differences in the pharmacokinetics and/or blood-to-brain equilibration rate of the compounds tested. The method produced highly reproducible results, with substantial advantages in terms of cost, turnaround time and animal welfare.
Comparison with existing methods: The results agreed with those reported in other, more elaborate pre-clinical models and in humans, enabling brain disposition to be assessed in a simple, efficient and robust *in vivo* model for new chemical entities.
Conclusions: Introducing the presented method in drug discovery allows brain disposition to be assessed earlier in the drug discovery pipeline and thus facilitate the selection of potent and penetrant CNS drugs.

Abbreviations: AUC, area under the plasma concentration curve; BBB, blood–brain-barrier; B/P-ratio, brain/plasma-ratio of drug concentration; CNS, central nervous system; IV, intravenous; PK, pharmacokinetics; PO, per os (oral); $t_{1/2}$, the terminal half-life of the drug plasma concentration; BBB PS_u, BBB permeability surface area product to unbound drug.

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http://dx.doi.org/10.1016/j.jneumeth.2014.04.022
0165-0270/

1. Introduction

To reach targets in the CNS, the blood–brain-barrier (BBB) is a key obstacle for small molecule therapeutics (Mensch et al., 2009), where as many as 98% of compounds intended for CNS therapeutics are blocked due to their inability to cross from the blood stream into the brain. Cost, resources and time are main drivers in the application of BBB assessments in drug discovery ranging from *in silico*, *in vitro* to *in vivo* and ultimately in human studies. Making *in vivo* assessment more affordable while maintaining high quality and robustness are necessary drivers for the development of better *in silico* and *in vitro* models (Pardridge, 1999) and will allow their introduction early in the lead optimization to pre-clinical phases of drug discovery (Goodwin and Clark, 2005).

In vivo brain penetration has been assessed in a great variety of experimental settings, some upon transient dosing: single oral (Sun et al., 2009), intravenous (Wang et al., 2013), or subcutaneous (Liu et al., 2005, 2012; Doran et al., 2005; Kalvass et al., 2007) and other performed at steady state using constant infusion in conscious animals (Summerfield et al., 2007).

Goodwin and Clark (2005) note that the partitioning between brain and blood measurements depend upon experimental conditions in each particular dosing regimen, and state that variations in the measured blood–brain partitioning for a given compound depends upon sampling time. This variability is due to differences in the PK and BBB equilibration (Brodie et al., 1960) of each compound tested, which is an inherent difficulty for any transient dosing regimen in particular in a drug discovery screening setup. Ideally for a steady state infusion setup the impact of variable blood–brain equilibration rates is removed and in practice it can be considerably reduced, depending upon how long the steady state is maintained.

On the other hand, obtaining steady state in animals may add complexity to the method, both in terms of turnaround time of the experiments, instrumentation and potential issues related to animal welfare. Here we present a simple model able to reach plasma steady state concentration in a one day triplicate experiment in anesthetized rats.

Comparing the presented method, which uses three animals, to the oral AUC method (Fortuna et al., 2013; Mittapalli et al., 2013) there is a significant reduction in number of animals used. Assessing brain penetration using the oral AUC method generally requires between three and seven sampling time points, which with a triplicate sampling scheme at each time point amounts to a considerable number of animals. Using the presented method would thus be a three- to sevenfold reduction.

2. Materials and methods

2.1. Chemicals

Naproxen (CID 156391, Sigma Aldrich), aricept (CID 3152, Sigma Aldrich), indomethacin (CID 3715, Sigma Aldrich), ketorolac (CID 3826, Sigma Aldrich), risperidone (CID 5073, Sigma Aldrich), ibuprofen (CID 3672, Sigma Aldrich) and ciglitazone (CID 2750, Tocris). Polisorbate 80 (Sigma Aldrich), polyethylene glycol 400 (Sigma Aldrich), NaCl solution 0.9% w/v (Eurospital), glucose solution 5% w/v (Galenica Senese). The formulation summary for each reference compound is described in the supplementary data. All chemicals used in the experiments were of the highest available grade. Dosing solutions were microfiltered for intravenous administration.

2.2. Animal experiments

Male Wistar Han Rats of 7–9 weeks age, weighing 250–275 g, were obtained from Charles River Laboratories – Calco (LC), Italy. Upon arrival, the rats were maintained for at least 5 days on a 12 h light/dark cycle in a temperature- and humidity-controlled environment with free access to food and water. All procedures were conducted in accordance with approved Siena Biotech Animal Care and Use Procedures and approved by Siena Biotech's Ethics Committee. Procedures and facilities were in compliance with the requirements of European Commission Directive 86/609/EEC concerning the protection of animals used for experimental and other scientific purposes. National legislation, harmonizing with this Directive, is defined in Decreto Legislativo no. 116 of 27 January 1992.

2.3. Experimental procedure

On the day of the experiment, 3 animals/compound were anesthetized (isoflurane), kept under inhalant anesthesia and thermoregulated (Homeothermic Blanket Control System Harvard mod HB 101/2) throughout the entire procedure. A catheter (BPVC T30) was inserted in the right jugular vein for blood sampling procedures. A vein catheter (22G) was inserted into the caudal vein for compound administration and test compounds were given as a single IV bolus injection (loading dose) followed by a slow IV infusion for 4.5 h (infusion dose) using pumps (KD Scientific KDS-100-CE) with a fixed dose volume (5 mL/kg).

Blood was collected from the jugular vein at the following time points: 5 min, 1.5, 2.5, 3.5, 4 and 4.5 h. At each time point, 200 μL of blood were obtained, collected in a Lithium Heparin tube (Sarstedt Multivette® 600 LH) and stored on ice. Catheter patency was ensured by flushing the catheter with saline/heparin 25 IU solution after each bleeding. All samples were centrifuged at 3220 *g* for 15 min at 4 °C within 20 min from sampling and then stored at −80 °C.

After the last blood sampling, while still under anesthesia, the animals were sacrificed using cervical dislocation; the head was removed using surgical bone scissor with a cut immediately after the occipital part of the skull (*forame magnum*). Using scissors, a midline incision was made in the skin and the skin was flipped over the eyes to free the skull. With small surgical scissors (Iris scissors) the skin was removed from the top of the skull starting from the occipital part at the point of the parietal bone.

Using surgical bone scissors a small cut was made through the *forame magnum* on the left and on the right of the occipital part of the skull and with curved narrow pattern forceps the interparietal bones were broken off; subsequently the skull was opened from the parietal to the frontal part at the point of the parietal bone, making sure not cutting through the brain. Both sides of the parietal and frontal bone were tilted with a curved narrow pattern forceps. Particular care was taken managing the meninges, which surround the brain, to ensure they did not cause ruptures in the brain while breaking off the skull to excise the brain. The meninges were gently removed with forceps. When the brain was freed from the meninges, a surgical spatula was slid under the anterior part of the brain (*olfactory bulb*) and the brain was gently tilted upward. To break the optic nerves and other cranial nerves, the spatula was slid further down and the brain was gently lifted out of the skull and collected in dedicated tubes (Precellys Lysing Kit 7 mL) and kept on ice. In this manner the brains were collected from *cerebellum* to the *olfactory bulbs*.

A subgroup of animals dosed with naproxen were flushed *via* trans-aortical perfusion with ice-cold saline/heparin 25 IU solution