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Comparison of the Effects of Perfusion in Determining Brain Penetration (Brain-to-Plasma Ratios) of Small Molecules in Rats

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In the process of drug discovery, brain and plasma measurements of new chemical entities in rodents are of interest, particularly when the target receptors are in the brain. Brain-to-plasma ratios (B/P) obtained from a rodent pharmacokinetic assay are useful in helping determine which compounds are brain penetrant. The study reported here was performed to determine whether whole-body saline perfusion for complete blood removal was required to accurately measure brain tissue compound concentrations. Diazepam was used as a positive control since it is highly brain penetrant. Compound A was used as a negative control since it had known poor brain penetration. After intravenous dosing with either diazepam or compound A, rats were anesthetized and blood was collected, then the brain was removed following no perfusion or whole-body perfusion with saline. The analytes described (compound A, diazepam, and the internal standard) were recovered from plasma or brain homogenate by use of protein precipitation, and were subsequently analyzed by use of liquid chromatography/tandem mass spectrometry (LC/MS/MS). The B/P values determined by use of LC-MS were not significantly different in perfused vs. non-perfused rats ($P \geq 0.05$). This approach (whole brain collected from non-perfused male rats) is an attractive alternative over brain penetration studies of perfused rats, since it has markedly reduced the technical time and potential for pain and distress required for generating B/P data due to elimination of the requirement for anesthesia and surgical preparation of animals.

The blood-brain barrier (BBB) serves to restrict movement of solutes between blood and brain, therefore maintaining a constant neuronal microenvironment (6). It protects the brain from foreign substances, endogenous hormones, and certain neurotransmitters in the blood that may harm the brain. The BBB does not generally allow large molecules or highly charged molecules into the brain. There are congenital, environmental, traumatic, and infectious circumstances that break down the BBB and result in serious complications, such as meningitis or encephalitis, by allowing typically restricted particles into the brain. However, there also are situations in which it is beneficial to gain access across an intact BBB. Medicines with certain physical properties that facilitate crossing the BBB can be developed to treat specific CNS diseases. For instance, diazepam is able to rapidly cross into the brain due to its highly lipophilic nature (7). It is one of the most commonly prescribed drugs for treating status epilepticus (4), and was introduced as an anxiolytic sedative drug more than 30 years ago.

We have used a rodent model, known at our facility as a rodent brain penetration assay, in which drug is delivered intravenously to rats, and terminal brain and plasma samples are obtained at specific time points. Data generated from these studies are then used to select compounds for testing in slower throughput efficacy rodent models. The correlation between high drug concentration in the brain/plasma penetration studies and superior efficacy in a relevant rodent model can help in selecting lead pharmaceutical compounds for specific CNS-regulated diseases or syndromes.

Due to the potential for blood contamination from the normal brain vasculature (1, 2), we previously perfused the brain prior to analysis. However, the procedures involved with brain perfusion were time consuming. In addition, the results were variable due to the inability to take tissue specimens at exact time points during harvesting. This variability was principally a result of animal-to-animal variability in the time required to reach an appropriate surgical anesthesia depth and was less a result of technical variability during the surgical procedure. There also was an underlying concern that anesthesia would interfere with the metabolism of a given drug, therefore affecting the accuracy of the data generated.

It was believed that the amount of blood contamination should not significantly affect the drug concentration due to the large volume of the brain tissue and small volume of blood in the brain. The study reported here was undertaken to compare the differences in drug concentrations, using perfused and non-perfused brains following drug administration with either a highly brain penetrant (positive control) or a poorly brain penetrant (negative control) agent. Diazepam was selected as a positive control due to its lipophilic nature and high brain penetrant characteristics. Compound A, a spiroindane-based peptidomimetic proprietary compound, synthesized at Merck Research Laboratories and previously evaluated in our brain penetration rat protocol (data not shown), was selected as a low brain penetrant agent.

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Materials and Methods

Chemicals. Diazepam (MW = 284.7) and polyethylene glycol (PEG-400) were purchased (Sigma Chemical Co., St. Louis, Mo.). Dehydrated 200-proof ethyl alcohol (EtOH) was purchased from Pharmco Inc. (Brookfield, Conn.). Acetonitrile (HPLC grade) was obtained from Fisher Scientific (Fair Lawn, N.J.). Compound A (MW = 518) and an internal standard (MW = 582) were synthesized in the Department of Medicinal Chemistry at Merck Research Laboratories, Rahway, N.J.

Animal preparation. Naïve, male Sprague-Dawley rats (300 to 350 g, Taconic Farms, Germantown, N.Y.) were studied. Prior to study, they were group housed, and were provided with rodent chow (Teklad 7012, Madison, Wis.) ad libitum and reverse osmosis water via an automatic watering system. The light:dark cycle was 12:12 h, and the building was provided with HEPA-filtered air maintained at 22 ± 1°C. The rodent colony was determined to be specific pathogen free, as indicated by results of gross necropsy, endo- and ectoparasite examinations, and the Charles River Laboratories (Wilmington, Mass.) Assessment Plus serology rat profile of sentinel animals. After overnight food restriction, rats were administrated 1 mg of diazepam or compound A per kilogram of body weight intravenously via the tail vein. Diazepam and compound A were formulated at a concentration of 1.0 mg/ml in EtOH:PEG400:water (10:40:50, vol:vol:vol) and were sterile filtered (0.2 μ*M*) prior to dosing. At each sampling time point (15 min, 1 h, 2 h), rats (n = 5 per time point; 2 groups: perfused or non-perfused after blood collection, 3 time points per group) were either euthanized with an overdose of inhalant CO_2 or were anesthetized with 4% isoflurane (Baxter, Deerfield, Ill.). Blood samples were collected by cardiocentesis.

Whole brain was collected from euthanized rats without perfusion or from anesthetized rats following saline perfusion. The perfusion procedures were as follows: After midline thoracotomy, the left ventricle was catheterized by use of a 23-gauge butterfly needle (Becton Dickinson, Franklin Lakes, N.J.). The right atrium was incised, the descending aorta was clamped, and the brain was perfused with sterile saline (0.9%, Abbott Laboratories, North Chicago, Ill.) at the rate of 10 ml/min for 3 min. All collected samples remained at –80°C until analysis. All animal procedures involved the humane care and use of animals, were performed within an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), International-approved facility, and were approved by the Merck-Rahway Institutional Animal Care and Use Committee (IACUC).

Extraction procedure. Each brain was washed with distilled water and weighed. Brains were homogenized using a Biohomogenizer (Biospec Products, Bartlesville, Okla.), with four equivalents of 0.1*N* sodium carbonate (4 ml per gram of brain tissue) in 20-ml scintillation vials. Internal standard solution prepared in 50:50 acetonitrile:water (100 μl) was added to plasma and brain aliquots (50 and 100 ml, respectively). Standard and control samples were made daily by adding 25 μl of the corresponding stock solution to blank plasma and blank brain homogenate aliquots. Standard curves for brain and plasma were generated at 5 to 5,000 ng/g and 2 to 2,000 ng/ml ranges, respectively. After preparation of standards, quality control samples, and unknowns, 0.2 ml of acetonitrile was added to the sample (plasma or brain homogenate) tube, which then was vortexed. Another 0.5 ml of acetonitrile was added, and the sample was vortexed again. The samples were centrifuged at 1,550 ×*g* for 5 min. The supernatant was transferred into a clean vessel, and was evaporated under an N_2 stream for 30 min. Mobile phase (0.2 ml of 60% acetonitrile, 5 m*M* ammonium formate, 0.1% formic acid) was added to the dried extracts, which then were transferred to an LC autosampler for analysis.

The LC/MS/MS conditions. Mass spectrometry was carried out, using an API 3000 triple quadrupole mass spectrometer (MDS Sciex, Concord, Ontario, Canada) operating in positive ion mode attached to two Series 200 micropumps (Perkin Elmer, Shelton, Conn.), and a Series 200 autosampler (Perkin Elmer). Quantitation was performed in the multiple reaction monitoring (MRM) mode following optimization of mass spectrometry (m/z) conditions for diazepam and compound A. For diazepam, the transitions of m/z 285 → 154 and 285 → 193 were monitored. For compound A, the transition of m/z 519 → 259 was monitored. The internal standard was monitored, using the transition of m/z 583.5 → 152. The response factors for transitions were optimized by adjustments of collision energy, declustering potential, and focusing potential.

Statistical analysis. A statistical model that accounts for the three factors of method (perfusion, no perfusion), time (15, 60, and 120 min), and compound (diazepam, A) was fit to the data. The logarithmic scale was used to better satisfy assumptions of normality and constant variance, and to naturally express comparisons as percentage differences. All subsequent comparisons used $P = 0.05$ to claim statistically significant differences. Graphs of individual data points used jittering to alleviate overlap without distortion of overall patterns. All calculations were performed in the R software environment (1996) (3, 5).

Results

This study was undertaken to compare differences in drug concentrations using perfused and non-perfused brains after drug administration with either a highly brain penetrant (positive control) or a poorly brain penetrant (negative control) agent. Diazepam was selected as a positive control due to its lipophilic nature and high brain penetrant characteristics. Compound A, a proprietary compound synthesized at Merck Research Laboratories, was selected as a low brain penetrant agent. Figures 1 and 2 represent the results for rat brain drug concentrations at various time points after intravenous administration of diazepam and compound A. Results between the perfused and non-perfused drug concentrations at various time points were in good agreement. Tables 1 and 2 represent average plasma and brain concentrations for diazepam and compound A in perfused and non-perfused animals. The B/P values for diazepam at all time points were consistently greater than two (2.0), indicative of its high brain penetration. When drugs have low brain penetration, B/P values are typically less than 0.2 (9). For diazepam, we found high brain concentration (500 ng/g at 15 min) and high B/P (2:1) in rats treated with or without perfusion during tissue harvesting. Although plasma concentrations of compound A and diazepam were similar, the brain concentration of compound A was relatively low compared with the brain concentration of diazepam. The data analysis indicated that results of perfused and non-perfused brain penetration experiments were not significantly different ($P \geq 0.05$). This approach suggests that a simple procedure (brain collection without perfusion) can be applied for measurements of analyte in rat

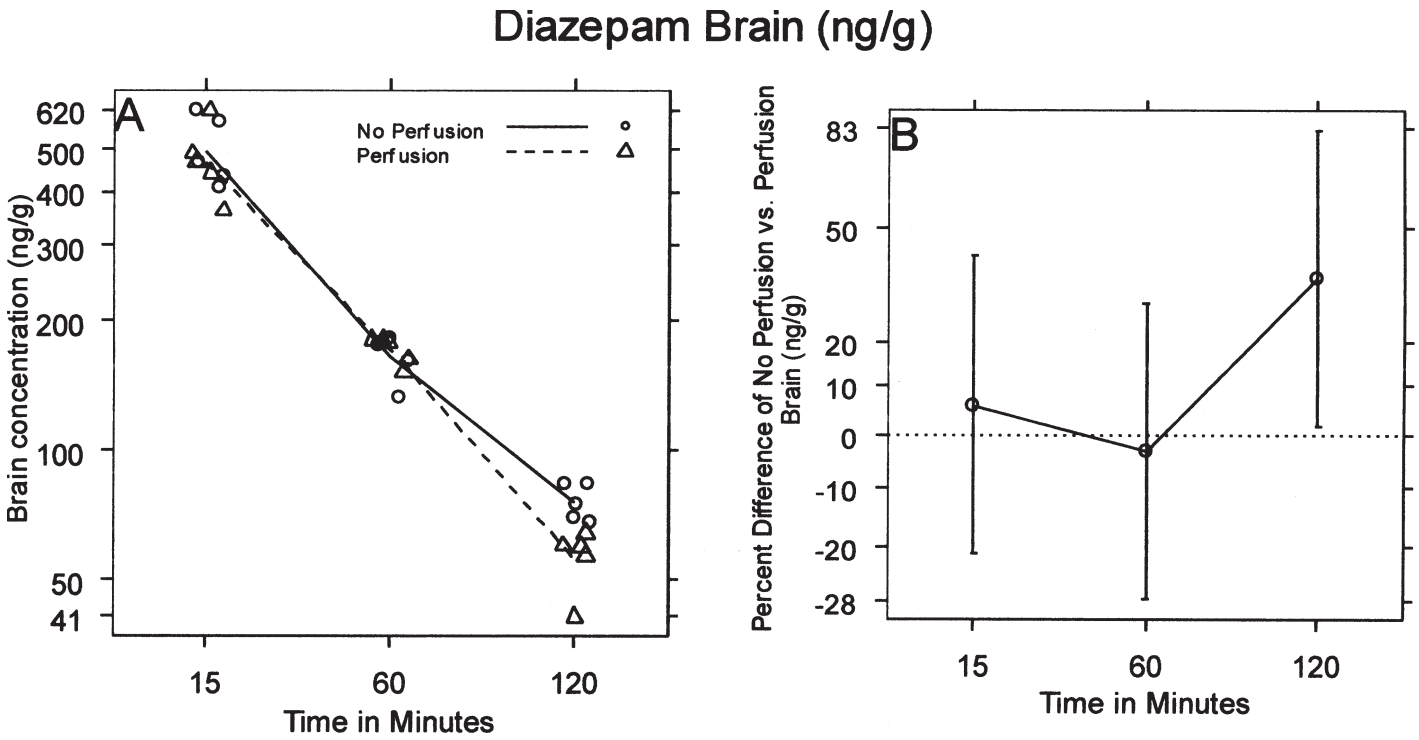

Figure 1. (A) Graph of the brain data from individual animals for the two collection methods, with mean profile lines added. (B) The 95% confidence intervals on the average difference of perfusion vs. no perfusion. The clear overlaps with zero indicate that the methods are not significantly different ($P > 0.05$) for the 15- and 60-min time points. The 120-time point suggests a marginally statistically significant increase under the no perfusion method.

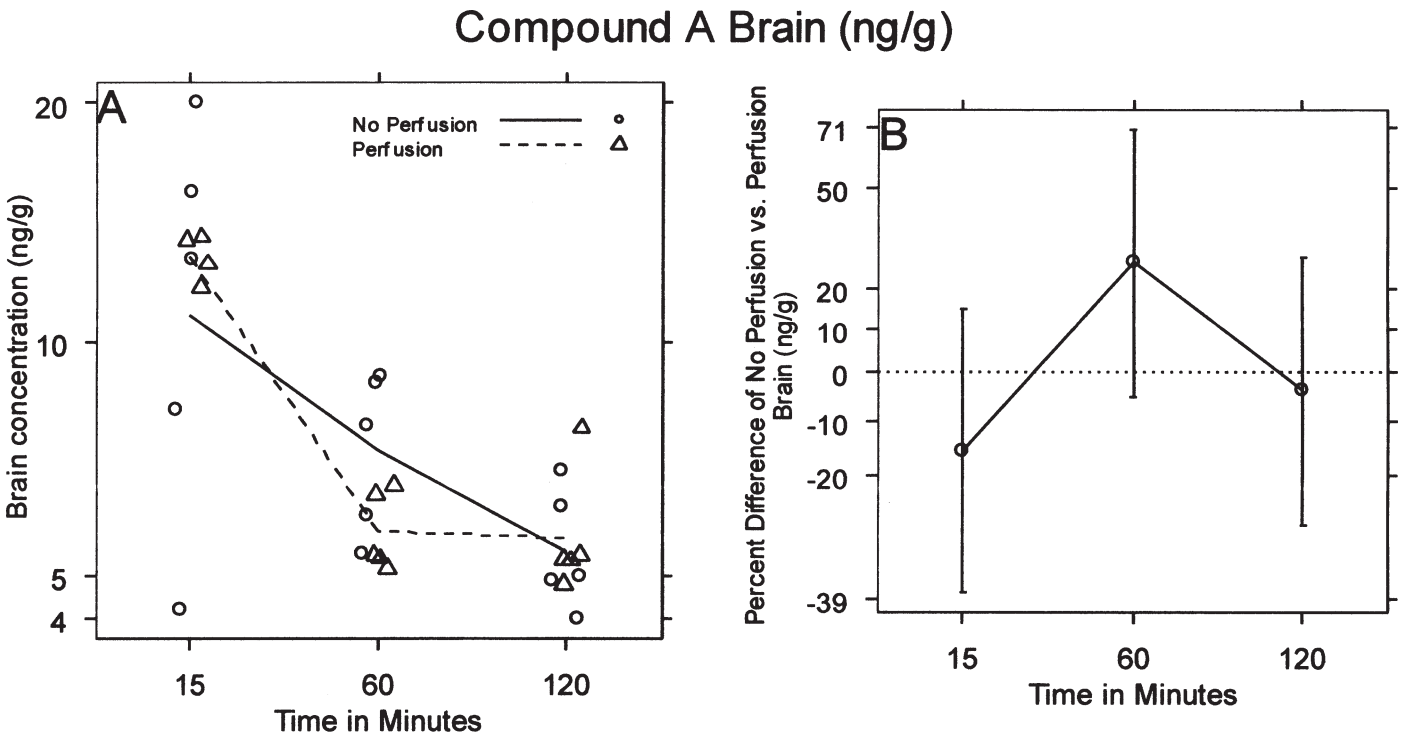

Figure 2. (A) Graph of individual plasma data for the two collection methods, with mean profile lines added. (B) The 95% confidence intervals on the average difference of perfusion vs. no perfusion. The overlaps with zero indicate that the methods are not significantly different ($P > 0.05$) for any of the time points.

Table 1. Brain and plasma concentrations of diazepam in rats after intravenous administration

	Brain (ng/g of brain tissue)		Plasma (ng/ml)		B/P	
Time (min)	Perfused	Non-perfused	Perfused	Non-perfused	Perfused	Non-perfused
15	467.5 ± 48.2	495.2 ± 51.0	170.5 ± 22.5	187.9 ± 24.8	2.74 ± 0.31	2.64 ± 0.30
60	170.7 ± 17.6	165.2 ± 17.0	58.9 ± 7.8	44.3 ± 5.8	2.90 ± 0.33	3.73 ± 0.42
120	55.6 ± 5.7	75.6 ± 7.8	16.3 ± 2.2	19.0 ± 2.5	3.41 ± 0.39	3.97 ± 0.45

B/P = Brain-to-plasma ratio.
Data are expressed as mean ± SEM.

Table 2. Brain and plasma concentrations of compound A in rats after intravenous administration

	Brain (ng/g of brain tissue)		Plasma (ng/ml)		B/P	
Time (min)	Perfused	Non-perfused	Perfused	Non-perfused	Perfused	Non-perfused
15	12.9 ± 1.5	10.9 ± 1.1	194.1 ± 29.0	189.5 ± 25.0	0.07 ± 0.01	0.06 ± 0.01
60	5.7 ± 0.6	7.2 ± 0.7	46.5 ± 6.1	58.7 ± 7.8	0.12 ± 0.01	0.12 ± 0.01
120	5.6 ± 0.6	5.4 ± 0.6	31.0 ± 4.1	39.0 ± 5.1	0.18 ± 0.02	0.14 ± 0.02

See Table 1 for key.

brain. This animal procedure is an accurate and rapid method that facilitates today's high throughput screening.

Discussion

These results support the supposition that brain penetration and plasma comparison studies can be performed without perfusion of the brain prior to analysis when compounds with either high or low brain penetration are evaluated. Many benefits can be derived through elimination of the perfusion step. The Early Discovery process in the pharmaceutical industry benefits greatly from time-saving methods of identification. Without the perfusion step, we are able to increase the number of compounds being screened by markedly reducing the technical time and surgical expertise required for each study. However, as a compound moves later into the Discovery process, perfusion may be needed with use of compounds of low penetration where we absolutely want to rule out contribution of drug concentrations in the brain attributable to blood contamination. Additionally, some level of verification that this practice works when evaluating a novel class of compounds should be performed. In consideration for improving animal welfare, we are always trying to identify methods to reduce or eliminate the potential for pain in animal-based research. Because animals destined for euthanasia are being used, we are able to avoid the potential for pain and distress through elimination of surgery. As scientists, we strive to improve the quality of our data. By eliminating the perfusion step, the time points for sample collection are more accurate and consistent between animals and studies, making the data more reliable. Therefore, we have been able to address one of Russell and Burch's three Rs (8): refinement. Results from similar studies (data not shown) have provided us with additional evidence on the validity of this approach.

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