Validation of the Triple-Tracer Autoradiographic Method in Rats

Hirofumi Nakai, MD, Mirko Diksic, PhD, and Y. Lucas Yamamoto, MD, PhD

We have developed a quantitative autoradiographic technique to measure simultaneously local cerebral blood flow, tissue pH, and local cerebral glucose utilization using 4-[18F]fluorooantipyrine, [14C]dimethyloxazolidine-2,5-dione, and 2-[3H]deoxyglucose as radioactive tracers. Our technique was validated by measurements of local cerebral blood flow, tissue pH, and local cerebral glucose utilization in the brains of sham-operated rats. Sham-operated rats showed a 14% reduction in local cerebral blood flow and a 10% reduction in local cerebral glucose utilization in the most affected piriform and entorhinal cortical regions compared with the contralateral homologous regions. There was no difference in tissue pH measured in the sham-operated side compared with normal rats or with the contralateral region. (Stroke 1988;19:758-763)

Materials and Methods

Theoretical Consideration

Triple-tracer autoradiography is based on the different physical half-lives and energies of three radionuclides used as labels ([18F]-FAP, [14C]-DMO, and [3H]-2-DG). Since radionuclide contamination in autoradiograms is <4%, subtraction is not required.

The first differentiation was performed by employing 5 mCi of a radionuclide with a short half-life ([18F]FAP) and only 25 µCi of a radionuclide with a long half-life ([14C]-DMO and [3H]-2-DG). Since radionuclide contamination in autoradiograms is <4%, subtraction is not required.

The second differentiation was obtained by placing a 5 μm-thick polyester layer (Mylar) between brain sections and x-ray film to absorb low-energy [14C]radioactivity, providing a pure [18F]FAP image, confirmed by the absence of an image of [3H] standards. The third differentiation was obtained by incubating brain sections in acetone dimethyl acetal (ADA) for 12 hours to remove [14C]-DMO. After incubation in ADA, the residual [14C]radioactivity resulted in <3% contamination of the [3H]autoradiogram.

General Procedure

Wistar rats (200–250 g), fasted except for water for 14–16 hours, were used in the experiments. The rats were anesthetized initially with 1.5–2.0% halothane, with topical application of 2% lidocaine jelly to all wound sites during cannulation of the femoral artery and vein and during subtemporal craniectomy for the sham operation. The rats were allowed to awake from anesthesia, and the lower half of the body was immobilized with a loose-fitting plaster cast on a lead block. Blood pressure and blood gases were serially checked during the experiments. Body temperature was kept at approximately 37°C with a heating pad.

Time Course of Tracer Administration

Rats received three different tracers and doses (5 mCi of [18F]-FAP, 25 µCi of [14C]-DMO, and 600 µCi of [3H]-2-DG) according to the following time schedule: wake up at 0 hr, [14C]-DMO at 1 hr, [3H]-2-DG at 2 hr 15 min, [18F]-FAP at 2 hr 59 min, and decapitation at 3 hr. Two hours elapsed between administration of the first
tracer, ⁴C-DMO, and killing of the rat. To reduce experimental error, the times for administering all tracers and for killing the rats were kept as constant as possible.

**Effect of Acetone Dimethyl Acetal**

The effects of incubation in ADA on deoxyglucose and deoxyglucose-6-phosphate in brain sections was evaluated by injecting 25 μCi of ⁴C-2-DG into a rat. The rat was killed 45 minutes later, and the brain was sectioned into 20-μm layers. Adjacent sections were separated into two groups, one with and the other without 12 hours’ incubation in ADA. Representative sections from both groups underwent protocol digestion, and ⁴C radioactivity of the solutions was measured by a liquid scintillation counter. The effect of ADA on ⁴C-2-DG radioactivity was evaluated by comparing optical densities of these two groups as well as the total tissue ⁴C radioactivity in representative sections.

The effects of incubation in ADA on ⁴C-DMO in brain sections was evaluated by injecting a rat with 25 μCi of ⁴C-DMO. The rat was killed 2 hours later, and the brain was sectioned into 20-μm layers. Adjacent sections were separated into two groups, one with and the other without 12 hours’ incubation in ADA. Representative sections from both groups underwent protocol digestion, and ⁴C radioactivity of the solutions was measured by a liquid scintillation counter. The fraction of ⁴C-DMO radioactivity remaining in brain sections after incubation in ADA was estimated from these results.

**Preparation of ¹H Standards**

Since ¹H emits low-energy β particles (E_v = 5.7 KeV), self-absorption for ¹H is greatly influenced by the nature of tissue. It has been reported that self-absorption for ¹H in white matter is almost double that in gray matter. Hence, correction for regional differences in self-absorption of the ¹H autoradiogram (tissue quenching) using an organic solvent is a promising solution to this problem.

To recalibrate commercial methacrylate ¹H standards (Amersham Corp., Arlington Heights, Illinois) as well as to account for the change in tissue density after incubating in ADA, nine doses of ³H-2-DG ranging from 25 to 500 μCi were injected intravenously into rats. The rats were killed 45 minutes later, and their brains were removed and sectioned. One group of 20-μm sections was collected on coverslips after incubation for 12 hours in ADA and used for scintillation counting. The other group was collected on glass slides and incubated in ADA for 12 hours and then exposed to ³H-sensitive x-ray film (LKB Ultrofilm, LKB-Producent, Bromma, Sweden) for 3 weeks along with methacrylate ¹H standards (Amersham). The optical density–tissue radioactivity relation was evaluated in the prepared ¹H standards, giving us a cross-calibration of methacrylate ¹H standards. (Details of the method as well as pertinent data are available from the authors on request.)

The effect of ADA incubation on tissue ¹H concentration estimates was evaluated by preparing brain homogenate ¹H standards. Incubation of these ¹H standards in ADA for 12 hours resulted in 30% weight loss due to lipid extraction. This reduction in the lipid content resulted in a 30% increase in the optical density due to a decrease in the self-absorption. Total tissue content of ¹H-2-DG and its metabolite was estimated from the linear relation of the radioactivity of ¹H tissue standards and the optical density. A log–log plot (Figure 1), as suggested by Gray et al., was used. (Actual data are available from the authors on request.)

**Autoradiographic Procedure**

Brains were removed from the skulls, frozen in liquid Freon-12, and cut coronally into 20-μm sections in a cryostat (American Optical Company, Buffalo, New York) at -22°C. Three consecutive sections were cut for each structure, collected on glass slides, and dried at room temperature. An ¹F-FAP autoradiogram (first exposure) was performed by immediate 2-hr exposure of the brain sections and ¹F standards, prepared as described elsewhere, together with ¹C and ¹H methacrylate standards to Kodak SB-5 x-ray film (Rochester, New York). Cross-contamination of ¹F with ¹C was <4% in pathophysiological conditions.

To prevent evaporation of ⁴C-DMO after the first exposure, brain sections were stored in a refrigerator at 4°C. Three days (39 ¹F half-lives) after the first exposure, a ⁴C-DMO autoradiogram (second exposure) was made for 3 weeks at 4°C. To absorb low-energy β particles emitted by ¹H, Mylar was placed between Kodak SB-5 x-ray film and the brain sections along with ¹C and ¹H methacrylate standards. Since there was no photographic density produced from ¹F or ¹H standards in the second exposure, it was assumed that the autoradiographic images from the second exposure were entirely due to ¹C.

To obtain the ²H-2-DG image, ¹C tissue radioactivity had to be removed from the brain sections. ²H-2-DG autoradiograms (third exposure) were obtained by exposing brain sections that had been incubated in ADA, along with ¹C and ¹H methacrylate standards, to LKB Ultrofilm for 3 weeks. Cross-contamination of ²H with ¹C in the third exposure was estimated by placing Mylar between LKB Ultrofilm and the brain sections. Photographic density was measured with a Photovolt densitometer (Model 52, Photovolt Co., New York, New York) equipped with a 0.1-mm aperture.

**Measurement of LCBF.** The method for measuring LCBF using ¹F-FAP is based on the ¹F-IAP autoradiographic technique described by Sakurada et al. At 44 minutes after injection of ²H-2-DG and at 2 hours after injection of ⁴C-DMO (see time course of tracer administration) 5 mCi of ¹F-FAP in 0.6 ml of physiological saline was injected intravenously for 1 minute as a constant infusion. The tissue–blood partition coefficient of 0.89 for ¹F-FAP was used.

**Measurement of tissue pH.** One hour after the rat woke up it was injected with 25 μCi of ⁴C-DMO in
0.25 ml of physiological saline as a bolus (see time course of tracer administration). At 1 and 2 hours after 14C-DMO injection, 20-μl arterial blood samples were taken, and radioactivity of the plasma was measured in a scintillation counter. Arterial blood gases were measured approximately 2 hours after 14C-DMO injection. After this measurement, the rat was decapitated. The spinal cord was removed, homogenized using a mechanical and an ultrasound homogenizer, and prepared as standards to provide a cross-reference for each autoradiogram. Three days (39 18F half-lives) later, 14C concentration was measured in a liquid scintillation counter. A correction for quenching and cross-contamination with 3H was made by using appropriate channels as prescribed by the manufacturer of the counter (LKB Wallac, Turku, Finland). Brain tissue pH was calculated by the equation described by Arieff et al. The pH and DMO concentrations in the extracellular space were assumed to be equal to those in arterial plasma.

**Measurement of LCGU.** The method for measuring LCGU using 3H-2-DG is based on the 14C-2-DG autoradiographic technique described by Sokoloff et al. Six hundred microcuries of 3H-2-DG in 0.6 ml of physiological saline was injected intravenously for 1 minute as a constant infusion. A procedure essentially the same as that used previously in our laboratory was carried out in measuring LCGU.

**Results**

A linear relation between the logarithm of optical density and the logarithm of tissue radioactivity (mCi/mg) is shown in Figure 1. The air-dried tissue standards and those incubated in ADA are equivalent, and they differ from the values given by Amersham as tissue equivalents. An excellent linear relation also indicates that there was no significant loss of radioactivity from brain sections by incubation in ADA.

Physiological variables for the sham-operated rats are given in Table 1. No significant differences were observed between these rats, rats with MCA occlusion, or normal rats in our laboratory.

A representative triple-tracer autoradiogram (Figure 2) shows LCBF, pH, and LCGU at selected anatomic locations in sham-operated rats. Table 2 presents numerical values of LCBF, LCGU, and local cerebral glucose utilization:blood flow ratios (LGFR) measured in both the sham-operated and nonoperated sides of the same rats after awakening. The mean ± SD LGFR in 12 selected structures was 0.78 ± 0.11 (Table 2).

A 14% reduction in LCBF and a 10% reduction in LCGU were observed in the most affected areas of the sham-operated side except for one region that is very close to the operative field (Figure 3, Table 3). Despite this reduction, LGFR remained within the normal range (Table 3). Tissue pH in the sham-operated side was generally lower (e.g., granular cortex and caudate nucleus) than in homologous regions of the nonoperated side or in normal brain. However, the decrease was not significant (Table 3).

**Discussion**

Five millicuries of 18F-FAP (2.8 mCi at exposure time), 25 μCi of 14C-DMO, and 600 μCi of 3H-2-DG were injected into all rats. Preliminary experiments revealed that up to 30 nCi 3H/mg brain tissue did not produce any significant darkening on Kodak SB-5 x-ray...
TABLE 2. LCBF, LCGU, and LGFR in Three Sham-Operated Rats

<table>
<thead>
<tr>
<th>Structure</th>
<th>LCBF (ml/100 g/min)</th>
<th>LCGU (μmol/100 g/min)</th>
<th>LGFR (μmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nonoperated side</td>
<td>Sham-operated side</td>
<td>Nonoperated side</td>
</tr>
<tr>
<td>Visual cortex</td>
<td>125 ± 12</td>
<td>111 ± 16</td>
<td>103 ± 6</td>
</tr>
<tr>
<td>Auditory cortex</td>
<td>161 ± 16</td>
<td>150 ± 6</td>
<td>121 ± 5</td>
</tr>
<tr>
<td>Parietal cortex</td>
<td>178 ± 21</td>
<td>157 ± 13</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>Sensorimotor cortex</td>
<td>182 ± 25</td>
<td>167 ± 22</td>
<td>101 ± 7</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>174 ± 20</td>
<td>177 ± 12</td>
<td>102 ± 9</td>
</tr>
<tr>
<td>Thalamus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lateral nucleus</td>
<td>119 ± 17</td>
<td>110 ± 7</td>
<td>95 ± 1</td>
</tr>
<tr>
<td>Ventral nucleus</td>
<td>108 ± 18</td>
<td>98 ± 8</td>
<td>79 ± 8</td>
</tr>
<tr>
<td>Medial geniculate</td>
<td>132 ± 22</td>
<td>132 ± 29</td>
<td>96 ± 7</td>
</tr>
<tr>
<td>Mammillary body</td>
<td>120 ± 10</td>
<td>121 ± 8</td>
<td>104 ± 11</td>
</tr>
<tr>
<td>Caudate nucleus</td>
<td>112 ± 17</td>
<td>97 ± 3</td>
<td>93 ± 9</td>
</tr>
<tr>
<td>Globus pallidus</td>
<td>70 ± 15</td>
<td>61 ± 8</td>
<td>52 ± 7</td>
</tr>
<tr>
<td>Inferior colliculus</td>
<td>141 ± 10*</td>
<td>145 ± 8</td>
<td>125 ± 14</td>
</tr>
<tr>
<td>Pontine gray matter</td>
<td>101 ± 13</td>
<td>102 ± 11</td>
<td>53 ± 9</td>
</tr>
<tr>
<td>Cerebellar cortex</td>
<td>76 ± 18</td>
<td>71 ± 3</td>
<td>53 ± 2</td>
</tr>
<tr>
<td>Cerebellar nucleus</td>
<td>119 ± 16*</td>
<td>110 ± 12</td>
<td>83 ± 4</td>
</tr>
<tr>
<td>Corpus callosum</td>
<td>33 ± 3</td>
<td>30 ± 1</td>
<td>32 ± 4</td>
</tr>
<tr>
<td>Internal capsule</td>
<td>37 ± 5</td>
<td>35 ± 4</td>
<td>33 ± 3</td>
</tr>
<tr>
<td>Mean ± SD for 12 structures</td>
<td></td>
<td></td>
<td>0.78 ± 0.11</td>
</tr>
</tbody>
</table>

Values are mean ± SD. LCBF, local cerebral blood flow; LCGU, local cerebral glucose utilization; LGFR, local cerebral glucose utilization/blood flow ratio. There was no significant difference between sham-operated side values and those reported by others in normal rat brain (LCBF4-16 and LCGU6-16). Tissue pH on sham-operated side did not differ from that in normal rat brain. There is no significant difference between LCBF and LCGU of nonoperated and sham-operated sides.

*p<0.05 different from conscious rat, two-tailed t test.

film during a 2-hour exposure (18F image). In the LCBF autoradiogram (first exposure), cross-contamination of 18F with 14C was <4% in pathophysiological conditions described by Sako et al16 and us (unpublished data). This cross-contamination (approximately 4% increase in 18F-assigned radioactivity from 14C in tissue) will result in an increase of approximately 21% in LCBF, which is substantially lower than rat-to-rat variability. Since 5-μm-thick Mylar prevents the energy of 3H from reaching the Kodak SB-5 x-ray film (proved by an

FIGURE 2. Triple-tracer autoradiograms in sham-operated rats at selected anatomic structures, using 4-[18F]fluoroantipyrine for measuring local cerebral blood flow (A–F), [14C]dimethylxazolidine-2,5-dione for tissue pH (G–L), and 2-[1H]deoxyglucose for local cerebral glucose utilization (M–R). Region of relative hypoperfusion compared with contralateral hemisphere is present around rhinal fissure, corresponding to position of craniectomy (A–F). However, no severe decrease of tissue pH (G–L) or severe change of glucose metabolism (M–R) exist.
FIGURE 3. Triple-tracer autoradiograms of sections of rat brain using (left) 4-[14F]fluoroantipyrine, (center) [14C]dimethyloxazolidine-2,5-dione, and (right) 2-[3H]deoxyglucose 3 hours after sham operation. Values for (left) local cerebral blood flow, (center) tissue pH, and (right) local cerebral glucose utilization are ml/100 g/min, no unit, and μmol/100 g/min, respectively, by region in Table 3. Capital letters indicate nonoperated side; lower-case letters indicate sham-operated side. A and a, frontal cortex; B and b, parietal cortex; C and c, granular cortex; D and d, agranular insular cortex; E and e, caudate nucleus (lateral portion).

absence of 3H standards image), the tissue pH autoradiogram (second exposure) originated solely from 14C-DMO. When 5-μm-thick Mylar was placed between 14C standards and LKB Ulstrofilm x-ray film, optical densities produced on the film by 14C standards were reduced. However, the linear relation between radioactivity and optical density was preserved (results not shown). Tissue 14C concentration was measured relative to the calibrated 14C methacrylate standards.

An independent autoradiogram resulting from 3H-2-DG and representing LCGU was obtained after removal of 14C-DMO by incubating brain sections in ADA for 12 hours. The loss of deoxyglucose radioactivity during incubation in ADA was assessed by preparing two groups of brain sections after injection of 14C-2-DG. 14C-2-DG rather than 3H-2-DG was used to eliminate self-absorption. One group of brain sections was incubated in ADA and exposed to x-ray film along with adjacent slices not incubated in ADA. There was no significant difference in optical density. Therefore, we concluded that there is no significant loss of 2-deoxyglucose or 2-deoxyglucose phosphate from brain sections during incubation in ADA. Completeness of 14C-DMO removal was assessed by sectioning rat brain after injection of 25 μCi of 14C-DMO. Liquid scintillation counting showed that <5% of the 14C-DMO remained in the tissue after incubation in ADA. However, autoradiograms showed only background darkening of the film (no image was seen). Therefore, we concluded that there is no significant contribution of 14C-DMO to the 3H image that resulted from 2-deoxyglucose. Cross-contamination of the 3H-2-DG images with 14C-DMO was estimated by exposing brain sections after 14C-DMO removal, with and without Mylar. Calculation showed that cross-contamination, calculated in nanocuries per gram, was <3%. This cross-contamination would result in a <3% increase in calculated LCGU (see Operational Equation 1 in Evans et al). LCBF, pH, and LCGU in the nonoperated side were compared with those previously reported using the same tracers and single-tracer autoradiography.4,5,7,16 There was no significant difference on either the sham-operated or nonoperated side except for LCBF in the inferior colliculus and the cerebellar nucleus (Table 2), in which there is a significant difference when compared with data reported by Sako et al.1 This can be explained simply on statistical grounds because of

<table>
<thead>
<tr>
<th>Region</th>
<th>LCBF (ml/100 g/min)</th>
<th>Tissue pH</th>
<th>LCGU (μmol/100 g/min)</th>
<th>LGFR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonoperated side</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>151 ± 24</td>
<td>6.91 ± 0.05</td>
<td>97 ± 4</td>
<td>0.64 ± 0.09</td>
</tr>
<tr>
<td>Parietal cortex</td>
<td>194 ± 35</td>
<td>6.94 ± 0.04</td>
<td>102 ± 6</td>
<td>0.54 ± 0.09</td>
</tr>
<tr>
<td>Granular cortex</td>
<td>142 ± 24</td>
<td>6.95 ± 0.03</td>
<td>112 ± 29</td>
<td>0.80 ± 0.17</td>
</tr>
<tr>
<td>Agranular cortex</td>
<td>130 ± 24</td>
<td>6.94 ± 0.03</td>
<td>109 ± 18</td>
<td>0.85 ± 0.20</td>
</tr>
<tr>
<td>Caudate nucleus (lateral portion)</td>
<td>130 ± 13</td>
<td>6.97 ± 0.02</td>
<td>110 ± 22</td>
<td>0.86 ± 0.20</td>
</tr>
<tr>
<td>Sham-operated side</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>134 ± 24</td>
<td>6.90 ± 0.03</td>
<td>91 ± 11</td>
<td>0.69 ± 0.15</td>
</tr>
<tr>
<td>Parietal cortex</td>
<td>175 ± 29</td>
<td>6.91 ± 0.04</td>
<td>95 ± 12</td>
<td>0.54 ± 0.05</td>
</tr>
<tr>
<td>Granular cortex</td>
<td>121 ± 11</td>
<td>6.91 ± 0.04</td>
<td>101 ± 18</td>
<td>0.85 ± 0.20</td>
</tr>
<tr>
<td>Agranular cortex</td>
<td>95 ± 9</td>
<td>6.90 ± 0.04</td>
<td>88 ± 8</td>
<td>0.87 ± 0.08</td>
</tr>
<tr>
<td>Caudate nucleus (lateral portion)</td>
<td>112 ± 4</td>
<td>6.95 ± 0.03</td>
<td>105 ± 19</td>
<td>0.94 ± 0.05</td>
</tr>
</tbody>
</table>

Values are mean ± SD. LCBF, local cerebral blood flow; LCGU, local cerebral glucose utilization; LGFR, local cerebral glucose utilization:blood flow ratio.
the relatively few sham-operated rats. Our results for mean LGFR of 12 selected structures (given in Table 2) do not differ significantly from those of Sako et al. 10

The loss of 2-deoxyglucose from ADA incubation described here is compatible with that of Diemer and Rosenørn, 11 who reported no effect on 3H-2-DG distribution in brain sections after an incubation in 2,2-dimethoxypropane (DMP) (1 minute × 2). However, Ginsberg et al 17 reported that >30% of the 14C-2-DG was lost from brain sections subjected to continuous agitation on a shaker table for 2–5 days while being dipped in DMP. Our results on loss of 2-deoxyglucose during incubation in ADA differ from those of Ginsberg et al 17 because of differences in the incubation solvent, the length of incubation, and the amount of shaking.

Analysis of the effect of damage to ischemic tissue on LCBF, tissue pH, and LCGU could provide a sensitive indicator of tissue damage since changes in both these parameters have been associated with the degree of cell damage. Accordingly, tissue pH combined with LCBF and LCGU is probably a more reliable and more sensitive indicator of tissue damage than any single parameter alone. Moreover, tissue pH in the rat brain 18 is higher than in the human brain, and as a result, it is more easily detectable in the experimental situation. Therefore, we conclude that our triple-tracer method is reliable. Its application in the MCA occlusion rat model is described in another study. 13

Acknowledgments

We would like to express our thanks to the staff of the Division of Neurosurgery Research Laboratory and Medical Cyclotron for their help and to Ms. F. Lumia for typing the manuscript.

References


13. Lear JL, Jones SC, Greenberg JH, Fedora TJ, Reivich M: Use of 13C and 14C in a double radionuclide autoradiographic technique for simultaneous measurement of LCBF and LCM-


Key Words • autoradiography • cerebral blood flow • rats
Validation of the triple-tracer autoradiographic method in rats.
H Nakai, M Diksic and Y L Yamamoto

*Stroke*. 1988;19:758-763
doi: 10.1161/01.STR.19.6.758

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1988 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/19/6/758

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Stroke* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Stroke* is online at:
http://stroke.ahajournals.org/subscriptions/