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]fluoroantipyrine, [14C]dimethyloxazolidine-2,5-dione, and 2-[1H]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)

Middle cerebral artery (MCA) occlusion in rats provides researchers with one of the most commonly used focal ischemic models. The extent and degree of ischemia vary from rat to rat, introducing additional noise to the data. Using quantitative autoradiographic techniques for measuring local cerebral blood flow (LCBF) with [14C]iodoantipyrine ("C-IAP") or 4-[18F]fluoroantipyrine ("F-FAP"); measuring tissue pH with [14C]dimethyloxazolidine-2,4-dione ("C-DMO"); and measuring local cerebral glucose utilization (LCGU) with 2-[1H]deoxyglucose ("C-2-DG") or 2-[1H]deoxyglucose ("H-2-DG"); we have also become aware of the heterogeneity of local perfusion, acid-base balance, and local glucose consumption in ischemic brain tissue.

To remove rat-to-rat variability from the observed relation between LCBF and LCGU, several double-tracer autoradiographic techniques for the simultaneous measurement of LCBF and LCGU were developed. In our laboratory, extensive use of double-tracer autoradiography for the simultaneous assessment of LCBF and LCGU, LCBF and tissue pH, and LCGU and tissue pH provided us with a unique opportunity to study their interrelations in an ischemic model.

We describe a triple-tracer autoradiographic method that provides us with three independent and quantitative autoradiograms representing brain LCBF, tissue pH, and LCGU. The method was validated in sham-operated rat brain.

Materials and Methods

Theoretical Consideration

Triple-tracer autoradiography is based on the different physical half-lives and energies of three radionuclides used as labels ("F-FAP, "C-DMO, and "H-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 ("F: t1/2 = 110 min, Eγ = 240 KeV) and only 25 μCi of a radionuclide with a long half-life ("C), as Sako et al have described. 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 "H, providing a pure "C image, confirmed by the absence of an image of "H standards. The third differentiation was obtained by incubating brain sections in acetone dimethyl acetate (ADA) for 12 hours to remove "C-DMO. After incubation in ADA, the residual "C radioactivity resulted in <3% contamination of the "H 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 "F-FAP, 25 μCi of "C-DMO, and 600 μCi of "H-2-DG) according to the following time schedule: wake up at 0 hr, "C-DMO at 1 hr, "H-2-DG at 2 hr 15 min, "F-FAP at 2 hr 59 min, and decapitation at 3 hr. Two hours elapsed between administration of the first...
tracer, "C-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 "C-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 "C radioactivity of the solutions was measured by a liquid scintillation counter. The effect of ADA on "C-2-DG radioactivity was evaluated by comparing optical densities of these two groups as well as the total tissue "C radioactivity in representative sections.

The effects of incubation in ADA on "C-DMO in brain sections was evaluated by injecting a rat with 25 μCi of "C-DMO. The rat was killed 2 hours later, and the brain was sectioned into 20-μm sections. 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 "C radioactivity of the solutions was measured by a liquid scintillation counter. The fraction of "C-DMO radioactivity remaining in brain sections after incubation in ADA was estimated from these results.

**Preparation of H Standards**

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

To recalibrate commercial methacrylate "H standards (Amersham Corp., Arlington Heights, Illinois) as well as to account for the change in tissue density after incubating in ADA, nine doses of "H-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 cover glasses 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 "H-sensitive x-ray film (LKB Ultrofilm, LKB-Producent, Bromma, Sweden) for 3 weeks along with methacrylate "H standards (Amersham). The optical density–tissue radioactivity relation was evaluated in the prepared "H standards, giving us a cross-calibration of methacrylate "H standards. (Details of the method as well as pertinent data are available from the authors on request.)

The effect of ADA incubation on tissue "H concentration estimates was evaluated by preparing brain homogenate "H standards. Incubation of these "H standards in ADA for 12 hours resulted in a 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 "H-2-DG and its metabolite was estimated from the linear relation of the radioactivity of "H 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 "F-FAP autoradiogram (first exposure) was performed by immediate 2-hr exposure of the brain sections and "F standards, prepared as described elsewhere, together with "C and "H methacrylate standards to Kodak SB-5 x-ray film (Rochester, New York). Cross-contamination of "F with "C was <4% in pathophysiological conditions.

To prevent evaporation of "C-DMO after the first exposure, brain sections were stored in a refrigerator at 4°C. Three days (39 1/2 half-lives) after the first exposure, a "C-DMO autoradiogram (second exposure) was made for 3 weeks at 4°C. To absorb low-energy β particles emitted by "H, Mylar was placed between Kodak SB-5 x-ray film and the brain sections along with "C and "H methacrylate standards. Since there was no photographic density produced from "F or "H standards in the second exposure, it was assumed that the autoradiographic images from the second exposure were entirely due to "C.

To obtain the "H-2-DG image, "C tissue radioactivity had to be removed from the brain sections. "H-2-DG autoradiograms (third exposure) were obtained by exposing brain sections that had been incubated in ADA, along with "C and "H methacrylate standards, to LKB Ultrofilm for 3 weeks. Cross-contamination of "H with "C 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 LCFB.** The method for measuring LCFB using "F-FAP is based on the "C-IAP autoradiographic technique described by Sakurada et al. At 44 minutes after injection of "H-2-DG and at 2 hours after injection of "C-DMO (see time course of tracer administration) 5 mCi of "F-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 "F-FAP was used.

**Measurement of tissue pH.** One hour after the rat woke up it was injected with 25 μCi of "C-DMO in
0.25 ml of physiological saline as a bolus (see time course of tracer administration). At 1 and 2 hours after \(^{14}\)C-DMO injection, 20-\(\mu\)l 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 \(^{14}\)C-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 \(^{18}\)F half-lives) later, \(^{14}\)C concentration was measured in a liquid scintillation counter. A correction for quenching and cross-contamination with \(^{3}\)H 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 extra cellular space were assumed to be equal to those in arterial plasma. \(^{5,22,23}\)

**Measurement of LCGU.** The method for measuring LCGU using \(^{3}\)H-2-DG is based on the \(^{14}\)C-2-DG autoradiographic technique described by Sokoloff et al.\(^{4}\) Six hundred microcuries of \(^{3}\)H-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\(^{4,18}\) 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,\(^{24}\) or normal rats in our laboratory.\(^{16,18}\)

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\(^{6}\) (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.\(^{2}\) However, the decrease was not significant (Table 3).

**Discussion**

Five millicuries of \(^{18}\)F-FAP (2.8 mCi at exposure time), 25 \(\mu\)Ci of \(^{14}\)C-DMO, and 600 \(\mu\)Ci of \(^{3}\)H-2-DG were injected into all rats. Preliminary experiments revealed that up to 30 nCi \(^{3}\)H/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>
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<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>Mamillary 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</td>
<td>0.78 ± 0.11</td>
<td>0.78 ± 0.11</td>
<td></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 (LCBF 16 and LCGU 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, 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 al 16 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]fluorooxylin for measuring local cerebral blood flow (A–F), [13C]dimethylaminopyridine-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.

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FIGURE 3. Triple-tracer autoradiograms of sections of rat brain using (left) 4-[14]Ffluoroantipyrine, (center) [14]Cdimethyloxazolidine-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 Ultronfilm 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 23).

LCBF, pH, and LCGU in the nonoperated side were compared with those previously reported using the same tracers and single-tracer autoradiography.4-5-716 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><strong>Nonoperated side</strong></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><strong>Sham-operated side</strong></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.16

The loss of 2-deoxyglucose from ADA incubation described here is compatible with that of Diemer and Rosenørn,11 who reported no effect on H2-2-DG distribution in brain sections after an incubation in 2,2-dimethoxypropane (DMP) (1 minute x 2). However, Ginsberg et al17 reported that >30% of the C2-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 al17 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 critical pH value related to LCBF and LCGU that would indicate irreversible tissue damage in this ischemic model. Accordingly, tissue pH combined with LCBF and LCGU is probably a more reliable and more sensitive indicator of tissue damage since changes in tissue pH are closely related to both the severity and the duration of ischemia.26,27

From this data, we conclude that our triple-tracer method is reliable. Its application in the MCA occlusion rat model is described in another article.24

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References

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