A Simple Method for Quantification of Biotinidase Activity in Dried Blood Spot and Its Application to Screening of Biotinidase Deficiency

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YAMAGUCHI, A., FUKUSHI, M., ARAI, O., MIZUSHIMA, Y., SATO, Y., SHIMIZU, Y., TOMIDOKORO, K. and TAKASUGI, N. A Simple Method for Quantification of Biotinidase Activity in Dried Blood Spot and Its Application to Screening of Biotinidase Deficiency. Tohoku J. exp. Med., 1987, 152 (4), 339-346 — A simple and reliable method for quantification of biotinidase (EC.3.5.1.12) activity in dried blood spot was devised by a modification of the colorimetric screening test developed by Heard et al. (1984). The enzyme reaction and hemoglobin denaturation were carried out in a U-bottomed microplate. An aliquot of the reaction solution was transferred to a flat-bottomed microplate. After the coupling reaction was started, the absorbance was measured in situ by a microplate-reader. Both intra- and inter-assay coefficient of variation (CV) values were less than 10%. Biotinidase activity in dried blood spot showed a good correlation to that in serum ($r=0.912$, $n=8$). This method was applied in a pilot screening of 18,945 newborns in Sapporo City. No positive results have been obtained as yet.

Wolf et al. (1983) have recently clarified that late-onset multiple carboxylase deficiency is attributed to deficient activity of biotinidase (EC.3.5.1.12), which plays an important role in the recycling of biotin. They proposed that early detection of the disease and administration of pharmacologic doses of biotin are necessary to prevent physical and neurologic abnormalities of affected infants (Wolf et al. 1985; Heard et al. 1986).

On the other hand, a simple screening method for biotinidase deficiency was developed by Heard et al. (1984). In the test, normal biotinidase activity in a disc obtained from dried blood spot yields purple coloration resulting from the coupling reaction of the liberated p-aminobenzoate (PAB) from N-biotinyl-p-aminobenzoate (B-PAB), an artificial substrate of biotinidase. Each sample is

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then classified qualitatively as normal or other based on the color tone of the reaction mixture.

In the present paper, a simple and quantitative screening method for biotinidase deficiency is described with a modification of the above method, and the results of a pilot screening conducted in Sapporo City are described.

**MATERIALS AND METHODS**

**Specimens.** Newborn and infant specimens were blood impregnated filter papers which were sent to us for the Guthrie test. Other specimens were prepared by dripping each adult blood onto the Guthrie filter paper and drying up in air for several hours. All specimens were stored at 4°C, unless otherwise noted.

**Sampling.** Dried, blood-saturated discs (3 mm diameter) were punched out from the filter papers.

**Reagents.** B-PAB was synthesized by the method of Wolf et al. (1983). Biotinyl-N-hydroxysuccinimide was purchased from Polysciences Inc. (Warrington, PA, USA). PAB, N-1-naphthylethylenediamine dihydrochloride, ammonium sulfamate and trichloroacetic acid (TCA) were obtained from Kanto Chemical Co. (Tokyo); sodium nitrite from Nakarai Chemicals Ltd. (Kyoto).

**Apparatus.** An Inter Med microplate-reader, Immno Reader NJ-2000 equipped with a microcomputer system, was used.

**Procedures.** To each disc in a 96-well, U-bottomed microplate, 30 µl of the substrate solution containing 250 µmole of B-PAB, 50 mmole of phosphate buffer (pH 6.0) and 5 mmole of EDTA per liter was added. The microplate was covered and incubated without shaking for 16 hr at 37°C. The reaction was stopped by adding 50 µl of 1.84 M TCA and hemoglobin denaturation was completed by standing for 10 min. Fifty µl-portions of the transparent reaction solution were transferred easily to a flat-bottomed microplate by use of a multichannel micropipette. For the coupling reaction, 50 µl of each of the following reagents was added sequentially at 3 min intervals: 14.5 mM sodium nitrite, 43.8 mM ammonium sulfamate, and 3.86 mM N-1-naphthylethylenediamine dihydrochloride. After 10 min, the absorbance was measured at 540 nm. The biotinidase activity was calculated using a PAB calibration curve, which was prepared as follows: to a U-bottomed microplate well, 30 µl of PAB solution containing 50 to 150 µmole of PAB per liter in the same medium as the substrate solution, without B-PAB, were added. Subsequent operations were identical to those described above without the incubation step. Samples which showed abnormal activity were detected by a statistical analysis in each assay.

**RESULTS AND DISCUSSION**

**Hemoglobin denaturation**

After the addition of TCA, bulky coagulation of hemoglobin occurred within 10 min; neither centrifugation nor filtration were required to pellet the transparent colorless supernatant. However, TCA was to be dispensed carefully, because vigorous dispensing resulted in milky turbidity. Therefore, TCA was added slowly by use of a multichannel micropipette and the mixture was allowed to stand without mixing so as to prevent the turbidity.

**Incubation time**

A time course was shown in Fig. 1. An approximately linear relationship
existed between the absorbance and the incubation time up to 16 hr. We used a 16 hr incubation for routine analyses, but if necessary to determine more exact activity, the incubation time can be fixed at 6 hr in the middle range of the linear relationship.

**pH of the substrate solution**

Biotinidase had maximal activity in the pH range of 5.2 to 6.2 for B-PAB (Fig. 2), but pH around 6.0 is preferable for the assay because the solubility of B-PAB decreases with decreasing pH.

**Enzyme amounts**

A series of whole blood containing various amounts of biotinidase was prepared by replacing varied portions of the plasma with the same volume of saline. Biotinidase activity in dried blood spot obtained from this series was measured. A linear relationship was found between the activity and plasma content (Fig. 3).

**Color development**

The coloration reached a plateau within 2 min after the addition of the final reagent and did not change even after standing overnight. On the other hand, in the original method (Heard et al. 1984), the color intensity should be read within 30 min because of gradual disappearance of the color. This is due to the adsorp-
Fig. 2. Effect of pH on biotinidase activity.
Blood-saturated discs obtained from dried blood spots were incubated for 16 hr at 37°C in the substrate solution containing 0.05 M phosphate buffer of various pH values. Each point represents the mean of 8 normal samples.

Fig. 3. Effect of the amounts of enzyme in the assay system.
Biotinidase activity in dried spots of blood samples in which different portions of plasma were replaced with the same volumes of saline was measured for 16 hr incubation at 37°C and the results were expressed as a function of plasma content. Each point represents the mean of 2 normal samples.
A Screening Method for Biotinidase Deficiency

Interference

As the color development occurs in the presence of a free primary aromatic amino group, some therapeutic drugs in routine samples might interfere with the assay. Heard et al. (1984) investigated the effects of sulfonamides and other therapeutic drugs on the biotinidase assay and demonstrated that sulfonamides gave purple color in the absence of B-PAB, but the other drugs did not interfere with the color development at their concentrations which greatly exceeded the expected concentrations in blood. Our observations supported this conclusion. No increase in mean and maximum absorbance was detected in the group of neonates (n=56) who were being treated with therapeutic drugs at the time of blood sampling. Furthermore, there was no coloration in the B-PAB free assay of the samples (n=23) showing high absorbance (more than 0.35) in the routine assays.

Reproducibility

The intra-assay CVs were in the range of 2.8 to 3.2% for PAB solutions and 5.1 to 8.0% for dried blood spots, and the inter-assay CVs were in the range of 3.3 to 4.7% for PAB solutions and 6.4 to 8.0% for dried blood spots (Table 1). The CV for between blood spots obtained from the same blood samples was 6.4% (n=4).

Correlation between activities in serum and dried blood spot

As shown in Table 2, the biotinidase activity in dried blood spot determined by this method was well correlated to that in serum, which was measured by the method of Wolf et al. (1983).

Stability in dried blood spot

Dried blood spots stored at different temperatures were assayed for

<table>
<thead>
<tr>
<th>Sample</th>
<th>Intra (n=7)</th>
<th>Inter (n=7)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mean abs.</td>
<td>CV(%)</td>
</tr>
<tr>
<td>PAB solution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>0.134</td>
<td>2.8</td>
</tr>
<tr>
<td>100</td>
<td>0.263</td>
<td>2.8</td>
</tr>
<tr>
<td>150</td>
<td>0.386</td>
<td>3.2</td>
</tr>
<tr>
<td>Blood spot*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a</td>
<td>0.215</td>
<td>5.3</td>
</tr>
<tr>
<td>b</td>
<td>0.260</td>
<td>8.0</td>
</tr>
<tr>
<td>c</td>
<td>0.196</td>
<td>5.1</td>
</tr>
</tbody>
</table>

*Absorbance was measured for 16 hr incubation at 37°C using a blood disc.
biotinidase activity (Fig. 4). The activity was rapidly decreased during storage at 25 and 37°C, especially in the first 7 days, while it was fairly stable at -20°C even after 30 days. Consequently routine samples, which are stored at 4°C and tested within 7 days after collection, may lose some of the enzyme activity before assay. Nevertheless, the determined value will be accurate enough to detect positive samples in routin screening, if each sample is kept under similar conditions.

### Application to the screening

During a 1-year pilot study (Sep. 1985-Aug. 1986), 18,945 neonates born in Sapporo City were screened using this method. The biotinidase activity of normal neonates ($n = 7,910$) was normally distributed with the mean ± s.d. of

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**TABLE 2. Correlation of biotinidase activity in serum and dried blood spot**

<table>
<thead>
<tr>
<th></th>
<th>Normal adults</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood spot*</td>
</tr>
<tr>
<td>Range</td>
<td>4.77-8.35</td>
</tr>
<tr>
<td>Mean</td>
<td>6.12</td>
</tr>
<tr>
<td>s.d.</td>
<td>1.13</td>
</tr>
<tr>
<td>Corr. Coeff.</td>
<td>$r = 0.912$ (p &lt; 0.01)</td>
</tr>
</tbody>
</table>

* 6 hr incubation; pmole/min/disc.
† 30 min incubation; nmole/min/ml-serum.

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Fig. 4. Stability of biotinidase activity in dried blood spot.

After the storage of dried blood spot for indicated times at different temperatures, biotinidase activity was determined for 6 hr incubation at 37°C. The activity for each day was plotted as the percentage of the initial activity. Each point represents the mean of 6 normal samples.
3.05 ± 0.68 pmole/min/disc. If the activity was less than 1.0 pmole/min/disc, the measurements were repeated on additional discs taken from the original filter paper, and if the activity was still below 0.6 pmole/min/disc or the absorbance was below 0.05, another filter paper sample was requested. Repeat measurements of 38 samples (0.20%) were made, and another sample was requested of six cases (0.03%). All of the recall results were normal, and therefore, no infants with biotinidase deficiency have been identified as yet. The incidence of biotinidase deficiency was estimated to be 1 in 41,000 newborns (Wolf et al. 1985). As no such figure has been reported in Japan up to now, more extensive screening should be carried out.

Heard et al. (1986) pointed out some possible factors to explain the abnormalities appearing on the initial filter paper samples, but they did not elaborate on those in the case of repeat samples taken from the same infant. The factors include improper handling of the samples such as high temperature conditions due to poorly controlled air conditioning and, as we experienced, incomplete drying of the blood spot during collection, which will cause the considerable loss of biotinidase activity along with abnormal results of the Beutler test. Another factor to be considered is immaturity of biotinidase development. Heard et al. (1986) demonstrated that the mean biotinidase activity in serum of neonates was 53% of that of normal adults, and that the activity was positively correlated with gestational age. This idea is also supported by our findings that the mean activity of neonates was 79% of that of 1-month-old infants and 61% of that normal adults, but no significant differences were observed between low birth weight neonates and premature neonates (Table 3). A third explanation for the abnormalities may be the presence of impaired liver functions, as we encountered in a 1-month-old infant with a hepatic disease, who showed low biotinidase activity (0.34 pmole/min/disc). In this case, subsequent filter papers and serum assays revealed no biotinidase deficiency.

In conclusion, the proposed method proved to be sufficiently accurate and

### Table 3. Age-relation in biotinidase activity

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number</th>
<th>Biotinidase activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Range</td>
</tr>
<tr>
<td>Neonate (4–7 days)</td>
<td>7910</td>
<td>0.37–5.59</td>
</tr>
<tr>
<td>Low birth weight†</td>
<td>453</td>
<td>0.80–5.59</td>
</tr>
<tr>
<td>Premature‡</td>
<td>175</td>
<td>0.84–5.23</td>
</tr>
<tr>
<td>Infant (1 month)</td>
<td>402</td>
<td>1.46–5.39</td>
</tr>
<tr>
<td>Adult</td>
<td>8</td>
<td>3.90–4.68</td>
</tr>
</tbody>
</table>

* 16 hr incubation; pmole/min/disc.
† <2,500 g of birth weight.
‡ <36 weeks of gestational age.
simple to use, furthermore, it could be applied readily in wide scale neonatal screening of biotinidase deficiency.

References


