Rapid Electrophoretic Determination of Neuron-Specific Enolase Isoenzymes in Serum

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This assay procedure for each of the two neuron-specific enolases (αγ and γγ) and the non-neuronal enolase (αα) in serum involves two steps: electrophoretic separation of the three isoenzymes—αα, αγ, and γγ—on cellulose acetate, and bioluminescence measurement of total enolase activity. From these data, the activity concentrations (U/L) of the three isoenzymes in serum are calculated. Both measurement steps are based on the enzymatic activity of enolase and thus differ from the immunological methods currently in use, which require the availability of specific antibodies. The method is rapid (approximately 30 min for both steps) and requires only 10 μL of serum for the complete analysis. Studies of normal children and adults, and of patients suffering from neuroblastoma and small-cell lung cancer, show that it is suitable for clinical use. Furthermore, the fact that both neuron-specific isoenzymes of enolase can be systematically separated is an advantage over immunological techniques in determining isoenzyme patterns for pathological samples.

Additional Keyphrases: bioluminescence, electrophoresis, cellulose acetate, neuroblastoma, small-cell lung cancer, reference interval

Recently, there has been increasing interest in determining enolase (2-phospho-D-glycerate hydrolyase; EC 4.2.1.11) isoenzymes, particularly those containing γ-subunits (γγ and γγ), the so-called neuron-specific enolases (1, 2). Measurement of these isoenzymes is considered useful in the diagnosis and therapeutic efficiency assessment of diseases, such as small-cell lung cancer in adults (3–7) and neuroblastoma in children (2, 8–10). Techniques currently used to assay enolase isoenzymes—radioimmunoassay, immunoenzymology, and immunocapture (11–17)—require specific antibodies and purified isoenzymes for preparing standard curves. The assays often take long to perform and are only suitable for serial determinations. Furthermore, because methods involving only anti-γ antibodies (12–14, 16) do not distinguish between the αγ- and γγ-isoenzymes, any heterogeneity of these forms that may exist in pathological samples may be masked.

Enolase isoenzymes have been separated by electrophoresis on cellulose acetate and then determined colorimetrically after NADPH is generated in a series of coupled enzymatic reactions (1). However, because of its comparatively low sensitivity, this procedure is useful only for analyzing samples with high enolase activities, such as those found in extracts of tissues and tumors (1, 18, 19), but not for serum samples in which enolase activities are low.

Here we describe a method for precise and reproducible assay of enolase isoenzymes, including the neuron-specific enolases, in serum. First, the serum is electrophoresed on cellulose acetate plates. The separated enolase isoenzymes are detected through the fluorescence of NADPH, which is produced by a sequence of enzymatic reactions coupled to enolase activity. This step determines the percentages of the three enolase isoenzymes. Second, the total activity concentration (U/L) of enolase in serum is determined by measuring the bioluminescence of the ATP produced in another coupled reaction system. The activity of each of the enolase isoenzymes is then calculated.

Materials and Methods

Reagents and Samples

ADP, AMP, NADP⁺, 2-phosphoglycerate, hexokinase (EC 2.7.1.1), glucose-6-phosphate dehydrogenase (EC 1.1.1.49, from yeast), and pyruvate kinase (EC 2.7.1.40, from rabbit muscle) were from Boehringer-Mannheim, Mannheim, F.R.G. Cellulose acetate plates (ISO Flur Titan III), electrophoretic buffer (Electra HR), and electrophoretic apparatus and integrator (Autoscaner Flur Vis) were from Helena Laboratories, Beaumont, TX. Trisacryl M-DEAE was from IBF, Villeneuve-la-Garenne, France. Disposable polystyrene minicolumns were purchased from Wright Scientific Ltd., Stonehouse, U.K. CNBr-activated Sepharose was from Pharmacia, Uppsala, Sweden.

Nonhemolysed serum samples were stored at 4°C and were analyzed within 24 h of collection.

Assay Procedures

Electrophoresis. For samples with total activity less than 31 U/L, we applied 1 μL at the cathodic side of the cellulose acetate electrophoretic plate. Samples with higher activity were diluted appropriately first. Electrophoresis was carried out in Tris barbital sodium buffer, pH 8.6–9.0 (one 18-g
packet of Electra HR buffer dissolved in 1.8 L of doubly distilled water) at 300 V for 7 min at room temperature. About halfway through the procedure (3 min before the end of migration), we prepared a "substrate" plate by impregnating a second cellulose acetate plate with 1 mL of a solution containing, per liter, 50 mmol of Tris HCl (pH 7.4), 2 mmol of MgCl₂, 20 mmol of KCl, 2 mmol of glucose, 90 μmol of ADP, 20 mmol of AMP, 0.5 mmol of NADP⁺, 1.2 mmol of phosphoglycerate, 4 kU of pyruvate kinase, 7 kU of hexokinase, and 3.5 kU of glucose-6-phosphate dehydrogenase. Just before use, to minimize diffusion, we dissolved sucrose in this reagent to give a final concentration of 300 g/L. After blotting the electrophoretic plate on filter paper to remove excess buffer, we placed it on the substrate plate, such that the two wet surfaces touched, then pressed the two plates together between two thick, prewarmed (37 °C) glass plates, and incubated at 37 °C for 20 min. At the end of the incubation, we separated the two cellulose acetate plates and promptly dried them with a hair dryer. The fluorescence of NADPH produced was stable under these conditions for at least 60 min. Enolase bands were visible in ultraviolet (256 nm). We measured the relative proportion of each of the bands by integrating the fluorescence tracings on the plates with the integrator. For a permanent record, we photographed the plates on Polaroid film, type 107.

Bioluminescence. We determined the total enolase activities of serum and of column eluates (see below), in 5-mL samples, according to a method described elsewhere (20). In this procedure, the phosphoglycerate formed from 2-phosphoglycerate by enolase is transformed into ATP in the presence of pyruvate kinase and ADP. We measured the rate of production of ATP with a bioluminometer (Bio-Counter M 2010 from Lumac, Basel, Switzerland), using a luciferin–luciferase reaction system.

Calculation of results. From the percentages of each isoenzyme determined after electrophoresis, and from the total activity concentration of enolase (U/L) measured by bioluminescence, we calculated: (a) the activity concentrations of each of the isoenzymes; (b) the activity due to the α-subunits (αα + αγ2), the “non-neuron enolase” activity; and (c) the activity due to the γ-subunits (γγ + αγ2), the neuron-specific enolase activity (21).

Procedures for Comparison and Identification

Ion-exchange separation of enolase isoenzymes. For ion-exchange chromatography, we used disposable minicolumns containing 0.5 mL of Trisacryl M DEAE equilibrated with buffer A (per liter: 10 mmol of Tris HCl and 5 mmol of MgSO₄, pH 9.0). To the column we applied 0.2 mL of serum diluted with 0.8 mL of buffer A, and then eluted the enolase αα with 1.5 mL of buffer A. Enolases αγ and γγ were then eluted sequentially from the column with 2.5 mL of buffer B (buffer A plus 120 mmol of KCl per liter) and 2.5 mL of buffer C (per liter: 100 mmol of Tris HCl, 5 mmol of MgSO₄, and 200 mmol of KCl, pH 7.0), respectively. Analytical recoveries of known quantities of the isoenzymes applied to the column varied from 90 to 102%.

Results and Discussion

Identification of Fluorescent Bands on Cellulose Acetate

Electrophoresis of human brain extract revealed three fluorescent bands. Their appearance depended on the presence of 2-phosphoglycerate in the substrate medium, which indicated that they possessed enolase activity (Figure 1, left, lane 1). Band 3 had a slow cathodic mobility, whereas band 1 showed a fast anodic migration. Band 2 had a slower anodic migration. We used two methods to identify the bands. For the first, we prepared αα, αγ, and γγ-isoenzymes from human brain extract by ion-exchange chromatography and used these as reference standards for comparison with the electrophoretic bands. This showed that bands 1, 2, and 3 corresponded to the αα, αγ, and γγ-isoenzymes respectively (Figure 1, left). In a second approach, we used immobilized anti-γγ antibody to selectively remove enolase isoenzymes containing γ-subunits from human brain extracts, then subjected the antibody-treated supernate to electrophoresis. This treatment left only band 3 (Figure 1, right, lane 2), which was identical to the αα-isoenzyme.

Figure 2 shows the separation of enolase isoenzymes in serum samples. We found the same three bands, αα, αγ, and γγ, as were previously identified in Figure 1. Again, the appearance of these bands was dependent on the presence of 2-phosphoglycerate in the substrate medium. In some serum samples, a faint fluorescent band (X) appeared between the αγ and γγ positions. When a sample of serum containing the three isoenzymes and the band X was treated with immobilized anti-γγ antibody, the γγ and αγ-isoenzymes were, as expected, selectively removed. The αα-isoenzyme and the (barely visible in the illustration) band X remained in the supernate (Figure 2, right, lane 2), indicating that the band X was not a γ-containing enolase isoenzyme. Further, this band appeared even in the absence of 2-phosphoglycerate, from which we conclude that it was not an enolase isoenzyme. Experiments in which ADP was omitted from the substrate medium indicated that the fluorescence of band X was also not due to adenylyl kinase (EC 2.7.4.3) activity. We did not further identify this band, because it was well separated from αγ and γγ and did not interfere in the estimation of enolase isoenzymes (Figure 3).

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**Fig. 1. Electrophoretic separation of enolase isoenzymes**

*Left*: Lane 1, human brain extract; lanes 2, 3, and 4, αα, αγ, and γγ-isoenzymes, respectively, prepared from human brain by ion-exchange chromatography.  
Right: lane 1, human brain extract; lane 2, human brain extract after treatment with anti-γγ antibody; 0, origin

**Fig. 2. Electrophoretic separation of enolase isoenzymes of human serum**

*Left*: lane 1, normal serum; lane 2, normal serum containing unidentified band X; lane 3, serum from small-cell lung cancer patient; lane 4, serum from patient suffering from recent head trauma.  
Right: lane 1, normal serum; lane 2, normal serum after treatment with anti-γγ antibody; 0, origin
Analytical Variables

Precision. To determine the precision of the electrophoretic step, we analyzed normal and pathological (above-normal activities of $\alpha\gamma$-isoenzyme) human sera 12 times on three different cellulose plates (Table 1). The CVs for the integration of the fluorescent bands in the normal ($\alpha\gamma$, 0.35 U/L) and pathological sera ($\alpha\gamma$, 3.3 U/L)—10% and 3.1%, respectively—compare favorably with other such isoenzyme determinations in clinical practice, e.g., creatine kinase and lactate dehydrogenase (23).

The precision of the bioluminescence step for determining total enolase activity in human serum with normal or low activity is summarized in Table 2. Day-to-day precision was determined with aliquots of pooled human serum, stabilized by dilution in a Tris acetate buffer (per liter: 100 mmol Tris acetate, 2 mmol of EDTA, 5 mmol of Mg acetate, and 0.5 g of sodium azide, pH 7.4), and stored at -20°C. Each day, one aliquot was thawed and used for enolase determination.

Lower and upper limits of assay validity. The linearity of bioluminescence determination has been described elsewhere (20). For a 5-μL sample, the lower and upper limits of validity were 0.2 and 10 U/L, respectively. Experiments with different dilutions of serum showed that the results of the fluorescence reaction used in the electrophoresis step varied linearly with enolase activity up to 31 U/L, either as total activity or for each of the three isoenzymes. Samples with greater activity than this were diluted appropriately before electrophoresis.

In the electrophoresis step it was difficult to measure accurately the low proportions of the two isoenzymes, $\alpha\gamma$ and $\gamma\gamma$, in the presence of the large proportion of $\alpha\alpha$ that is always found in serum (Figure 3). This limited the sensitivity of the assay. In our experience, the lower limit for accurate measurement of any of the isoenzymes in serum was 3% of the total enolase activity.

![Fig. 3. Densitometric integration curve of enolase isoenzymes from normal serum containing the unidentified band X](image)

| Table 1. Precision of Electrophoretic Determination of Enolase Isoenzymes |
|-------------------------------|----------------|----------------|
| Total activity, U/L           | $\alpha\alpha$ | $\alpha\gamma$ |
| Mean                          | SD             | CV, %          |
| 5.5                           | 93.5           | 0.6            |
| 9.2                           | 64.3           | 1.1            |
| n = 12 each.                  |                |                |

Comparison of the electrophoretic method with ion-exchange chromatography. We analyzed samples of human serum for enolase isoenzymes by the electrophoretic method as described above and by using ion-exchange chromatography as had been proposed previously (24, 25). A comparison of the results obtained by the two methods (Table 3) shows that they are in good agreement. In addition, the electrophoretic method takes much less time for completion of analysis (30 min) and a much smaller volume of sample (5 μL) than the chromatographic procedure does (0.2 mL of sample and an average of 3 h per analysis).

Enolase Activity in Sera

Normal sera. Normal values for adults were determined by use of samples from 34 blood donors (18 men and 16 women, ages 21 to 58 years). Values for children were obtained by using blood samples from patients (ages two months to 12 years) who were hospitalized for benign diseases and had normal routine clinical-chemical values. Total enolase and $\alpha\alpha$ and $\alpha\gamma$-isoenzyme activities were higher in children than in adults (Table 4), as has been reported (8, 10). We did not detect $\gamma\gamma$-isoenzyme in the serum of any of these subjects, although others report it. Using immunocapture by successive treatment with anti-$\alpha\alpha$ and anti-$\gamma\gamma$ antibodies and bioluminescence assay, Gerbitz et al. (17) found a mean enolase activity of 165 mU/L for adults. Ishiguro et al. (9), using immunoezymology, reported a value of 100 mU/L for children. Perhaps the discrepancy between these and our results is due to the fact that Gerbitz et al. used plasma in their study instead of serum as we have done.

Table 2. Precision of Bioluminescence Determination of Total Enolase Activity

<table>
<thead>
<tr>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
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<tr>
<td></td>
<td>Total activity, U/L</td>
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<td>5.0</td>
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Table 3. Comparison of Electrophoretic and Ion-Exchange Chromatographic Separation of Enolase Isoenzymes

<table>
<thead>
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<th>Sample no.</th>
<th>Total activity in undiluted sample, U/L</th>
<th>Electrophoresis</th>
<th>Chromatography</th>
</tr>
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<tr>
<td></td>
<td>$\alpha\alpha$</td>
<td>$\alpha\gamma$</td>
<td>$\gamma\gamma$</td>
</tr>
<tr>
<td>1</td>
<td>57.5</td>
<td>90</td>
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<td>12.8</td>
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</tr>
<tr>
<td>4</td>
<td>12.0</td>
<td>81</td>
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cases (all but no. 2 and 6). The activities of neuron-specific enolase were increased in all six cases. The maximum increases represented 7.1 times the upper limit of the normal reference interval for total activity, 5.5 times for \( \alpha \), and 174 times for neuron-specific enolase. The increase in neuron-specific enolase was mainly due to the \( \alpha \) form, \( \gamma \) being only moderately increased in three cases and not detected in the others. These findings of an increased neuron-specific enolase in sera of children with neuroblastoma agree with previous published data (2, 8–10). Ishiguro et al. (9) also noted the prevalence of \( \alpha \) and \( \gamma \) in 12 of their 16 cases.

Table 6 lists data for 10 cases of small-cell lung cancer. Total enolase and \( \alpha \)-isoenzyme activities were increased in all 10 (respectively, 1.8 to 9.7 and 1.6 to 6.0 times the upper limits of the normal range). The increases of neuron-specific enolase were more marked (from five- to 72-fold the normal limit) and involved mainly the \( \gamma \)-isoenzyme. The \( \gamma \)-isoenzyme was present in only one case, which also had the highest activities for total enolase and for \( \gamma \). These results are in agreement with published reports on the specific increase in neuron-specific enolase in sera from patients with small-cell lung cancer (3–7). However, the predominance of \( \gamma \)-isoenzyme that we found appears to contradict the findings of Gerbitz et al. (17), who in five of their six cases found more \( \gamma \) than \( \alpha \). We have confirmed the predominance of \( \gamma \) over \( \alpha \) by subjecting one of our samples to ion-exchange chromatography (Table 2). In any case, a strict comparison between the two studies is difficult, because the total enolase activity in most of the samples of Gerbitz et al. was higher than in our samples. Study of a greater number of samples may clarify this discrepancy.

In conclusion: the electrophoretic procedure described here has several positive features. It can be done quickly (less than 30 min for electrophoresis and less than 5 min for the bioluminescence measurement), and it requires less than 10 \( \mu L \) of serum for complete determination of all three isoenzymes. Results are obtained rapidly with a precision compatible with clinical requirements, and compare favorably with other, more-cumbersome techniques currently available. Furthermore, because it can be used for systematic analysis of all the abundant enolase isoenzymes found in serum (\( \alpha \), \( \gamma \), and \( \beta \)), we can investigate the clinical significance of any heterogeneity in isoenzyme composition observed in pathological sera.

This work was supported by grants from the Medical Research Council of Canada and Le Comité du Puy de Dôme de la Ligue Nationale contre le Cancer. We thank the France-Québec Exchange Program, which facilitated this collaborative research. We appreciate the technical assistance of Mme. N. Chardonnal.

References


