Rapid Assay of Lactate Dehydrogenase in Serum by the Stopped-Flow Method

KEN-ICHIRO KANAYA, KEITARO HIROMI*, MASAYUKI TOTANI** and TAKASHI MURACHI**

SUMMARY The stopped-flow method was applied to the emergent or urgent assay of lactate dehydrogenase in human serum by using a novel apparatus for microanalysis, which has newly been designed by one of the authors. The enzyme activity was easily estimated from the initial slope (velocity) of the reaction curve recorded at 340nm for only 5 s. Calibration curve was linear in the range of zero to 1000 IU/l. when 10 μl of serum was used, the detection limit was 30 IU/l and was comparable to that of conventional UV method. The coefficient of variation in within-run and that in between-run were <5% and <7% for the serums with the enzyme activity of >200 IU/l, respectively. Close correlation was found between the present method and the currently available UV method (r = 0.988).

Introduction

The assay of LDH (EC 1.1.1.27) in human serum is important in diagnosis of many diseases, such as myocardial infarction and hepatic diseases, as well as leukemia and certain other malignancies. Generally, LDH activity can be estimated by the methods based on the following reversible reaction\(^1,2\).

\[
\text{LDH} \\
\text{L-Lactate} + \text{NAD}^+ \rightleftharpoons \text{Pyruvate} + \text{NADH} + H^+
\]

Since, at neutral pH, the equilibrium is favored from right to left, it is more advantageous for the rapid assay of serum LDH to use pyruvate and NADH as substrates, and to follow the decrease in absorbance of NADH at 340 nm\(^3\). Several official methods for serum LDH adopt these procedures\(^4-6\). In this case, however, the measurements have to be performed in the presence of a large interfering background due to excess NADH, which
is often apt to cause serious errors in the absorbance measurement by using conventional spectrophotometers.

Recently, the stopped-flow method has widely been used in various fields including analytical chemistry. We have also developed several novel analytical procedures using the stopped-flow method, and have successfully applied them to the highly sensitive and/or highly selective determinations of ascorbic acid, reductone, phosphate, tryptophan, total protein and pyruvate. In addition to the principal characteristic of rapidity, it is an important advantage of the stopped-flow method that absorbance changes as small as 0.002 O.D. can precisely be measured even under large backgrounds up to about 2.0 O.D. By usual spectrophotometry, such minute absorbance change can hardly be determined with good precision, since errors due to irregular motion of cells or pulsatile flow of solution in flow-through cells are unavoidable. Thus, it can be expected that the stopped-flow method is useful to economize the reaction-monitoring period for obtaining a reliable progress curve for the reaction from NADH to NAD⁺, improving the rapidity of the assay.

In the present paper, a rapid method for the assay of human serum LDH is developed by using a novel micro stopped-flow apparatus, which needs only about 1/10 of the specimen amounts required by conventional macro apparatuses and is useful for saving precious specimens. The most important advantage of the proposed method is that merely 10μl of serum and a reaction-monitoring period as short as 5 s are sufficient for a reliable assay.

**Materials and Methods**

**Materials**

LDH as the calibrating standard (from pig heart, grade I) and NADH were purchased from Oriental Yeast Co. The sodium salt of pyruvate was obtained from Nakarai Chemicals.

**Preparation of substrate solution**

0.1 M phosphate buffer (pH 7.2) containing 3.2 mM pyruvate and 0.4 mM NADH was prepared as the substrate solution. The solution was degassed to prevent cavitation.

**Instrument**

A micro stopped-flow spectrophotometer of piston-driven and front-stopping type, which has newly been designed by Hiromi et al., was used to follow the time course of the reaction. By the apparatus, stable and precise monitoring of reactions could easily be done over a period of a few minutes. The construction of the apparatus is essentially the same as that of the commercially available apparatuses (macro apparatuses) used in our previous works. The main difference between the micro and macro apparatuses resides in the minimum necessary amounts of specimens. In the micro apparatus, the minimum specimen volume required for filling a flow-line and performing reliable measurement(s) without cavitation was only 100 to 200 μl, which is about 1/10 of the volume required by macro apparatuses. Other features of the micro apparatus are comparable to those of macro ones, as follows. The apparatus is equipped with two reservoirs. A couple of syringes are used for driving the solutions introduced from the reservoirs. Two pistons of the
syringes are rapidly and simultaneously driven by nitrogen gas pressure. Thus, two solutions can rapidly be mixed to commence a reaction. Immediately after the mixing, the flow is completely stopped. The optical change accompanying the reaction, which proceeds in the observation cell is continuously monitored. The temperature of the reservoirs and the cell can simultaneously be controlled by water-circulation. The optical path of the cell used was 10 mm. The dead time of the flow system was 3 to 5 ms under the conditions used. For further details of stopped-flow apparatuses, several review articles and a monograph may be referred to.

**Procedure**

Dilute 10 µl of serum (containing zero to 1000 IU/l of LDH) up to 200 µl with 0.1 M phosphate buffer (pH 7.2) and degas the resulting specimen solution. Put the specimen solution into one reservoir of the micro stopped-flow apparatus. Into the other reservoir, transfer the substrate solution. The reservoirs and the cell are maintained at 30°C. Mix the two solutions rapidly at a 1:1 ratio in the apparatus, and monitor the absorbance change at 340 nm continuously for 5 s as a function of time. Determine the initial velocity ($v_0$) as the slope (O.D./min) of the tangent line at the starting point of reaction curve; this way is generally known as "the initial slope method".

**Results**

*Concentration, pH and temperature*

Pyruvate and NADH concentrations (in the final solution after mixing in the apparatus) of 1.6 mM and 0.2 mM, pH of 7.2 and temperature of 30°C were all selected according to the conditions used by Vassault et al.2.

*Optimal monitoring period*

The minimum monitoring period required for developing a rapid assay with the accuracy and the precision comparable to those of the conventional methods was investigated and selected to be 5 s. A monitoring period of no longer than 1 s was too short to obtain suitable reaction curve for reliable measurement.

*Calibration Curve, detection limit and precision*

Typical reaction curves in the present method are shown in Fig. 1. Between $v_0$ determined from the reaction curves and LDH activity, good linearity was obtained as shown in Fig. 2. Each point in this figure represents the mean value of three determinations. Proportionality was obtained for LDH activity from zero to 1000 IU/l (1 IU/ml).

The reaction curves were obtained in the presence of a background as large as about 1.2 O.D. due to excess NADH. In spite of such a large interfering background as large as about 1.2 O.D. due to excess NADH.

![Fig. 1 Typical examples of reaction curves.](image_url)

The reaction curves were obtained in the presence of a background of about 1.2 O.D. due to excess NADH. LDH activity; A, O; B, 150; C, 300; and D, 600 IU/l.
Table I

<table>
<thead>
<tr>
<th>Serum</th>
<th>Within-run</th>
<th>Between-run</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>n</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Mean (IU/l)</td>
<td>206.8</td>
<td>547.4</td>
</tr>
<tr>
<td>SD (IU/l)</td>
<td>9.7</td>
<td>12.6</td>
</tr>
<tr>
<td>CV (%)</td>
<td>4.7</td>
<td>2.3</td>
</tr>
</tbody>
</table>

The assay method is described in Materials and Methods.

Fig. 2

Typical example of calibration curve. Each point represents the mean value of three determinations.

ground, the value of SD in v₀ determinations of the procedural blank was only 0.0015 O.D./min, which corresponded to about 10 IU/l of LDH in serum. The detection limit was decided to be 30 IU/l (±3×SD) in serum.

The results on the within-run and between-run variations for serums with LDH activity in the range of 200 to 800 IU/l are shown in Table I.

Comparison with a conventional method

The LDH activities in identical specimens from hospital population were simultaneously measured by the present micro stopped-flow UV method and by the conventional UV method using an autoanalyzer (a routine method at the Kyoto University Hospital). The correlation between these two methods was good (n = 25, r = 0.988) with the regression equation of the curve defined by y = 1.12x + 51.5, as shown in Fig. 3.
Discussion

A rapid assay method for human serum LDH was developed. The precision of the developed method is comparable to that of conventional methods, while the monitoring period of the former is significantly shorter (5 s) than that of the latter (usually 2 min\(^2,4\)\(^\text{--}\)\(^6\)). Thus, the proposed method is considered to be useful for emergent or urgent assay of serum LDH, especially for the real time assay at bedside.

The detection limit of 30 IU/l of the proposed method is comparable to that of conventional methods. If necessary, more sensitive determinations can be made by expanding the monitoring period. For example, with the monitoring periods of 30 and 120 s, the detection limits obtained by the present micro stopped-flow technique were 6 and 2 IU/l for serum LDH assay, which are about 1/6 and about 1/20 that of conventional methods with the monitoring period of about 120 s (2 min), respectively. Thus, the present micro stopped-flow technique keeps the capability for much highly sensitive assay of human serum LDH.

The saving of precious serums, especially from babies or emaciated patients, was greatly improved by using the micro stopped-flow apparatus. The minimum volume of serum required by the present method was only 10 \(\mu l\), while that by conventional methods is usually 50 to 100 \(\mu l\).

Thus, in this paper, the stopped-flow method is demonstrated generally to be useful for improving the rapidity and/or the sensitivity of "the initial slope method", especially with the measurements of absorbance changes in the presence of a large interfering background.

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References