Serum Succinate by Capillary Zone Electrophoresis: Marker Candidate for Hypoxia*

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ABSTRACT

Serum succinate may offer an alternate analyte to lactate for the evaluation of hypoxia. To evaluate the potential uses of succinate, a relatively rapid capillary zone electrophoresis assay was developed for use in the clinical laboratory setting. Employing a simple indirect ultraviolet detection method with commercially available instrumentation, the limit of detection for serum succinate was determined to be 0.1 \( \mu \text{mol/L} \), the upper limit of linearity 100 \( \mu \text{mol/L} \), and the between-run coefficient of variation about 15 percent. Based on specimens from 202 apparently healthy adults, the non-parametric reference interval was 1.0 to 9.2 \( \mu \text{mol/L} \). Preliminary studies in stored blood show succinate increased 2-fold while lactate increased 11-fold, suggesting that succinate may be a clinically useful marker for hypoxia in patients after blood transfusion. This assay provides a practical tool for the investigation of the clinical applications of succinate.

Introduction

Succinate is a major intermediate in the tricarboxylic acid cycle. Normal concentrations of succinic acid in the circulation are the result of sources such as leakage from mitochondria and uptake from the gut. It has been observed that underperfusion and hypoxia block the tricarboxylic acid cycle, deplete the tricarboxylic acid cycle intermediates in the mitochondria, and result in an elevated succinate concentration in the circulation. On this basis, succinate has been proposed as a marker for tissue hypoxia, similar to lactate. Where lactate is related to the glycolytic cycle, succinate is more closely related to mitochondrial metabolism. The circulating concentrations of both lactate and succinate reflect a balance from a variety of additional sources. For example, certain anaerobic bacteria excrete succinate, and the presence of succinate has been used as an indicator for such bacteria. Metabolic disorders can also alter the excreted and circulating succinate concentrations. Increased urinary succinate excretion can be found in conditions including maple syrup urine disease, multiple sclerosis, and glutaric aciduria. Studies on the clinical significance of succinate have been limited because of the relative
difficulty of the analysis. The traditional and still most widely used method is gas chromatography. Although it is accurate and precise, at the same time it is tedious and labor intensive, since it involves a lengthy derivatization step to form the alkyl ester or silyl derivative of the otherwise non-volatile acids. This derivatization step is not only difficult but time consuming. Other approaches include ion exclusion and high performance liquid chromatography, enzymatic methods, capillary electrophoresis, and even nuclear magnetic resonance.

Capillary electrophoresis is a technique which is simple and versatile, and emerging as a useful method for the clinical laboratory. An assay for serum succinate is described which uses commercially available, automated instrumentation suitable for routine analysis. The indirect detection method makes derivatization unnecessary, thus greatly simplifying the sample preparation procedure. The assay is currently being used to assess succinate as a marker for hypoxia after transfusion and in other conditions.

Methods

Cetyltrimethylammonium bromide (CTAB)* meso-2,3-dibromosuccinic acid (DBSA)† and other reagents‡ were purchased from specific companies. Capillary electrophoresis grade water§ was used to prepare all solutions. All the samples were analyzed on a 3D Capillary Electrophoresis instrument§ equipped with Chemstation software. Separation was achieved in uncoated fused silica capillaries with an effective length of 56 cm, an internal diameter of 75 μm, and an extended optical path bubble of 200 μm. The capillary was thermostated to a temperature of 25°C. The electrode polarities were switched, i.e., the detector was positioned at the positive electrode (anode). The instrument was operated in the constant current mode with a current value of 9 μA. Indirect UV detection was used with the signal of phthalic acid monitored at 210 nm against a reference signal at 320 nm. The monitoring and reference inputs were reversed, so the decreases in the phthalic acid background signal appeared as peaks rather than valleys (figure 1).

The stock electrolyte solution, with a concentration of 5 mmol/L phthalate and 0.7 mmol/L CTAB, was prepared with CE grade water. The pH of the solution was adjusted to 6.04 ± 0.01 with the addition of LiOH. The reconditioning electrolyte solution was prepared from the stock electrolyte solution by adjusting its pH between 7.1 and 7.2 with LiOH solution. The working electrolyte solution was made by mixing 70 mL acetonitrile and 130 mL stock electrolyte solution.

Preconditioning of new capillaries was done at 60°C by flushing the column with 1

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* Fluka Chemika, Buchs, Switzerland.
† Aldrich, Milwaukee, WI.
‡ Sigma, St. Louis, MO.
§ Hewlett Packard, Palo Alto, CA.
mol/L of NaOH for 10 minutes, then with 0.1 mol/L NaOH for 1 minute, followed by 0.1 mol/L LiOH for 5 minutes. The capillary was rinsed with CE grade water for 2 minutes, then with the reconditioning electrolyte for 10 minutes. The column was allowed to cool to 25°C. The initial drift of the capillary performance stabilized after approximately 5 normal runs. In each specimen run there was included a 3-minute preconditioning flush with the working electrolyte, and a 1.5-minute postconditioning rinse with the reconditioning electrolyte. After each sample, the inlet and outlet working electrolyte solutions were automatically replenished. At the end of the specimen batch, the capillary was rinsed and stored with preconditioning electrolyte.

Samples were prepared by first adding 20 μL of 500 μmol/L dibromosuccinate internal standard to 0.50 mL of serum specimen. Proteins were precipitated by the addition of 1 drop of concentrated hydrochloric acid followed by the addition of 2.0 mL of ethyl acetate. The mixture was vortexed for 30 seconds and then centrifuged for 5 minutes at 1300 × g on a Model 3000 E Mistral centrifuge. A minimum of 1.4 mL of the upper layer was separated, and the ethyl acetate was evaporated with nitrogen. The residue was redissolved in 0.20 mL of CE grade water, and the solution was centrifuged in a 10,000 molecular weight cutoff filter for 30 minutes at 2000 × g on a Model 5402 Eppendorf centrifuge to remove residual protein. The filtrate was then transferred into a loading vial of the instrument. The prepared sample was injected onto the capillary by a 25-second electrokinetic injection at 5 kV.

Calibration curves were obtained by adding 10, 50, and 100 μL of 50.0 μmol/L sodium succinate to a dialyzed serum pool (which contained no measurable succinate) to a total volume of 0.5 mL. The resulting 1, 5, and 10 μmol/L succinate solutions were extracted and analyzed as regular specimens. The calculation for theoretical plate number for succinate was calculated using the following equation.

\[
N = \frac{\mu_{\text{TOTAL}} V}{2D_0}
\]

where \(N\) is the theoretical plate number, \(\mu_{\text{TOTAL}}\) (in cm² V⁻¹ s⁻¹) is the total electrophoretic mobility, \(V\) (in volts) is the applied voltage, and \(D_0\) (in cm² s⁻¹) is the diffusion coefficient of the molecule. Lactate was determined by a routine lactate dehydrogenase method.

**Results**

An electropherogram of a typical patient serum specimen is shown in figure 1. Inorganic anions such as bromide, chloride, and nitrate elute first owing to their higher mobility, and, thus, do not interfere with the assay. The dibromosuccinate internal standard is well separated from succinate, there is baseline resolution between succinate and methylmalonate. Calibration curves showed a linear relationship between the area under the peak and the concentration of succinate in serum. A typical calibration curve showed a response of:

\[y = 0.269x + 0.0048, \text{ with } r = 0.9974.\]

The lower limit of detection, taken as 3 times the standard deviation of the blank serum pool, was found to be 0.1 μmol/L. Based on a series of spiked samples, the assay gave a linear response up to 500 μmol/L succinic acid. However, because of front tailing of the peak above 100 μmol/L, this was taken as the upper limit of linearity.

Precision was determined by first preparing a normal and a spiked serum pool. These two pools served as low and high controls. Two samples per run from each pool were run for 11 days. At the 4.8 μmol/L and at the 11.3 μmol/L levels, the within-run precisions were 12.6 and 14.8 percent, and the between-run precisions were 14.8 and 15.1 percent, respectively.

To address the effect of temperature on succinate stability, serum samples from healthy volunteers were stored at 4°C and −20°C, and the succinate concentrations checked regularly. In the refrigerated specimens, succinate concentrations decreased about 5 percent at 3 days and about 40 percent at one week. Frozen specimens remained stable during storage. However, repeated freeze-thaw cycles of standards, controls, and
specimens were observed to cause a significant loss of succinate (data not shown).

For the population study, 202 apparently healthy adults were selected (94 males and 108 females). The age dependence was addressed by plotting succinic acid concentrations against patient age. No correlation was found, the results being randomly distributed along the best fit line calculated by the least square regression method (data not shown). The histogram in figure 2 indicates substantial overlap between males and females. This was confirmed by the student t-test which showed no significant difference in succinate concentrations between the two populations (P = 0.055) at the 5 percent level of significance. The adult reference interval, established by the nonparametric method, i.e., excluding the top and bottom 2.5 percent of the population, was 1.0 to 9.2 μmol/L.

To study the accumulation of succinate in stored blood units, ethylenediamine tetraacetic acid (EDTA) whole blood from a healthy volunteer was stored at 4°C. The serum succinic acid as well as lactic acid concentration of this specimen was followed for two weeks. These conditions were taken to simulate storage of blood unit. An eleven-fold increase in serum lactic acid concentration was found while at the same time serum succinic acid increased less than two-fold (Table I).

**TABLE 1**

<table>
<thead>
<tr>
<th>Storage Day</th>
<th>Lactate mmol/L</th>
<th>Succinate μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.0</td>
<td>2.1</td>
</tr>
<tr>
<td>1</td>
<td>3.7</td>
<td>2.0</td>
</tr>
<tr>
<td>4</td>
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<tr>
<td>7</td>
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<td>2.4</td>
</tr>
<tr>
<td>14</td>
<td>11.0</td>
<td>3.9</td>
</tr>
</tbody>
</table>

EDTA = ethylenediamine tetraacetic acid.

**Discussion**

The theoretical plate number calculated for succinic acid was 200,000, a value which is not unusual in capillary electrophoresis separations. As with similar assays, proper conditioning of the capillary was essential for high quality resolution. Conditioning consisted of a preinjection flush with running electrolyte and was followed by a post-specimen flush to remove residual specimen components as

![Figure 2. Distribution of serum succinate values in an adult population.](image-url)
quickly as possible. These conditions were arrived at on an empirical basis. Resolution is also highly sensitive to changes in pH, and this factor needs careful quality control.

Anions such as citrate, malate, and methylmalonate, which migrate in the vicinity of succinate (figure 1), can potentially interfere with succinate when present at high concentrations. To date, a specimen has not been encountered where any of these compounds were present naturally at such a high concentration that they interfered with the assay. A number of specimens have been seen with markedly elevated methylmalonate concentrations (secondary to vitamin B12 deficiency) which did not interfere with the quantitation of succinic acid. However, citrated plasma could not be analyzed owing to the suppression of all the peaks by the high level of citric acid present. Hemolytic, icteric, and lipemic specimens do not interfere with the assay.

Because the population study demonstrated a significant skew to the distribution of serum succinate (figure 2), a nonparametric method was used to calculate the reference interval of 1.0 to 9.2 μmol/L. Age was not a significant variable, although the study was limited to an adult population. Although the statistical analysis did not show a significant difference between males and females, visual observation of the results suggests that women tend to have slightly higher succinate concentrations. The reason behind this observation is unclear. Whether this is an artifact of the relatively small population study remains to be determined.

Although lactate is a widely used marker for tissue hypoxia, there may be circumstances where other markers of hypoxia are also useful. For example, lactate concentrations increase significantly in banked blood units, and this reduces the usefulness of lactate as a marker for hypoxia after transfusion. Whereas lactate is a metabolic product of red blood cells, succinate is not. The results from a stored blood study summarized in table I are informative. The 11-fold increase of lactate concentration in two weeks suggests that when one unit of stored blood (about 0.5 L) is transfused, the lactate concentration in the circulation of the patient can potentially double. However, during the same time period the succinate in stored blood increases less than two-fold. Transfusion of one unit of blood would therefore add a clinically insignificant amount of succinate, suggesting that succinate may prove to be a useful marker for hypoxia in patients after transfusion. The possible clinical applications of measuring serum succinate are promising but will require further study to establish as an analyte of practical interest.

**Conclusion**

A novel capillary electrophoretic assay for serum succinate was developed and validated. The ease of performance and precision characteristics make the assay suitable for use in the clinical laboratory using commercially available, automated instrumentation. Serum specimens can be stored refrigerated for up to 3 days without significant loss of succinate, although specimens requiring longer storage should be promptly frozen. A preliminary population study indicates no gender or age dependency of serum succinate concentrations in adults, although women tend to have slightly higher values. Serum succinate may prove to be a useful marker of hypoxia, providing supplemental information to lactate. While the clinical utility of measuring serum succinate remains to be established, the assay described here provides a practical tool with which to investigate the uses of succinate analysis in the clinical setting.

**Acknowledgement**

A portion of this work was presented by GKH at the annual meeting of the Academy of Clinical Laboratory Physicians and Scientists, June 6, 1996, in St. Louis, Missouri (Am J Clin Path 1996;106:140).

**References**


2. Tarrach F, Herrmenn K. Organische Sauren der Gemusearten. IV. Die Veranderungen der Sauren


