A Kit for Citrate in Foodstuffs Adapted for Assay of Serum and Urine

Vilay S. Warty, Ronald P. Busch, and Mohamed A. Virji

A kit method for rapid analysis of citrate in foodstuffs, based on use of citrate lyase (EC 4.1.3.6), has been adapted and evaluated for use with clinical samples. Membrane filters removed serum proteins, which interfere with the analysis, and reagent and sample volumes were decreased from those in the manufacturer’s protocol, thereby decreasing the cost of the assay. Using gravimetrically prepared citrate standards, we determined that assay results varied linearly with concentration up to sixfold the upper reference limit for serum citrate. Intra- and interassay variation were within acceptable limits (CVs <3% and <8.5%, respectively). Kit reagents were stable for four weeks at −20 °C. Assay results were unaffected by hemolysis or other biochemical interferences. The method evidently provides a rapid, convenient microassay for citrate in clinical samples.

Additional Keyphrases: enzymic methods • “kit” methods

Determination of citrate in blood and urine is clinically useful in certain situations, such as during transfusions of very large amounts of blood and blood products to avoid citrate toxicity (1–3) and in assessing the risk of stone formation from measurements of citrate in urine (4). Methods for citrate determination in clinical samples have until recently been cumbersome, relatively nonspecific, and not amenable to adaptation for rapid analysis (5–7). In food sciences, a specific enzymic method involving bacterial citrate lyase (EC 4.1.3.6) has been used to determine citrate in various foods (8), and the principle of this technique has been applied to citrate analysis in clinical samples (9, 10).

In response to the needs of a liver-transplant program in pediatric and adult patients recently initiated at our institution, we adapted, and evaluated for analysis of citrate in clinical samples, a kit for citrate determination from Boehringer Mannheim, which had been used primarily with nonclinical samples.

The method of citrate assay is based on the enzymic conversion of citrate to oxaloacetate and acetate by citrate lyase, a bacterial enzyme from Aerobacter aerogenes. The enzyme preparation also contains oxaloacetate decarboxylase (EC 4.1.1.3), which decarboxylates to pyruvate part of the oxaloacetate formed with citrate with lyase (11). Hence, lactate dehydrogenase (EC 1.1.1.27) is included in the reaction mixture to convert pyruvate to lactate:

\[
\text{Citrate} \rightarrow \text{citrate lyase} \rightarrow \text{oxaloacetate + acetate} \\
\text{Oxaloacetate + NAD + H}^+ \rightarrow \text{malate + NAD}^+ \\
\text{Oxaloacetate} \rightarrow \text{oxaloacetate decarboxylase} \rightarrow \text{pyruvate + CO}_2 \\
\text{Pyruvate + NAD + H}^+ \rightarrow \text{lactate dehydrogenase} \rightarrow \text{l-lactate + NAD}^+
\]

The amount of NADH oxidized is stoichiometrically related to the amount of citrate present. The decrease in NADH is measured at 340 nm.

Materials and Methods

Materials

Citric acid dihydrate, trisodium salt, was obtained from Sigma Chemical Co., St. Louis, MO. The citric acid assay kit (cat. no. 119076), purchased from Boehringer Mannheim, Indianapolis, IN, consisted of two reagents. The first was a lyophilized powder containing malate dehydrogenase, lactate dehydrogenase, and NADH, which were reconstituted with 12 mL of distilled water. The second was lyophilized citrate lyase, which we dissolved in 300 μL of distilled water. All reagents were stored at −20 °C and used within four weeks after being reconstituted; the reagents were found to be stable over that period. "Centrifree"™ Microparticulation filters (cat. no. 4103/4104) were generously provided by Amicon Corp., Danvers, MA. With these filters, which have a molecular-mass cutoff of 40 000 Da, macromolecules can be removed rapidly from small samples without appreciable sample loss. Disposable plastic cuvettes for spectrophotometry were obtained from Bio-Rad Laboratories, Richmond, CA.

Reagents

Standards: Three concentrations of citric acid dihydrate (170, 850, and 2550 μmol/L) were prepared in de-ionized water and used as standards. Every four weeks we made new standards and stored them at −20 °C until used.

Controls: Pooled plasma from a blood bank was used as a control. In addition, gravimetric solutions of citrate in de-ionized water were prepared for aqueous controls.

Samples: Urine specimens from healthy individuals were kept frozen (−20 °C). Before analysis, the samples were brought to room temperature, then centrifuged at 1400 × g for 10 min in a table-top centrifuge to remove any particulate matter.

Procedure

Blood collected from healthy volunteers was allowed to clot at room temperature. The serum was separated, stored frozen (−20 °C), then equilibrated to room temperature before analysis. Walshman and McCambridge (9) and Tofftegaard (10) reported that serum had to be deproteinized before assaying for citrate; they used perchloric acid for protein removal, which required neutralizing the samples before analysis. However, we processed samples in a single step by using membrane filters that retain proteins above a certain molecular mass. We placed 1-mL aliquots in the sample reservoirs of the microparticulation filters, centrifuged these in a fixed-angle-rotor bench-top centrifuge at 350 × g for 15 min at room temperature, and used the filtrates for analysis.

We followed the protocol recommended in the assay kit insert, carrying out the reaction at room temperature (20–25 °C) at pH 7.8. We measured the change in absorbance at
340 nm and calculated the concentration of citrate from the decrease in NADH, using the molar absorptivity of NADH at that wavelength.

To adapt the assay for use with clinical samples, we halved the recommended amounts of samples and reagents, both for cost effectiveness and to reduce the sample-volume requirement for serum. Using serum/urine samples of 100 µL allowed us to make 36 analyses in duplicate per kit. Results for full-volume and half-volume assays of the three standards were similar (r = 0.999), so we used the smaller sample and reagent volumes in all subsequent assays.

Results

Analytical Variables

**Linearity**: We constructed a calibration curve by assaying in duplicate six citrate standards, ranging from 170 to 3400 µmol/L. Repeated analysis (n = 4) showed the curve to be linear up to 2700 µmol/L. Samples containing more citrate than 2250 µmol/L were diluted with de-ionized water (after filtration in the case of serum samples) and re-assayed.

**Precision**: Results for 112 patients’ serum samples analyzed in duplicate over a three-month period differed by 3.1% or less. Three aqueous controls and a pooled serum control were analyzed in duplicate for citrate over a three-month period with several runs and gave the results shown in Table 1.

**Recovery**: To determine analytical recovery, we added different concentrations of citrate (340–1360 µmol/L) to four aliquots of five serum samples from healthy volunteers and assayed them. Recovery of the added citrate ranged from 98.6% to 100.1%.

**Sample stability**: Pooled serum samples were analyzed for citrate content when freshly obtained, and subsequently at three-day intervals for five weeks after being stored at 4, −20, or −70 °C. Results for citrate in these samples were within the interassay variability range.

**Interference**: We studied three different categories of interferences:

a) **Filtered vs unfiltered serum and urine**: Proteins must be removed from serum samples because they may interfere (9, 10). In the present study, we used partition filters to prepare samples free of proteins with molecular mass >40 000 Da. We assayed, in the same run, 11 sera and eight urine samples, filtered and unfiltered. After filtration, all the serum samples showed significantly higher citrate values (p = <0.005, Student’s paired t-test), but there was no effect on the citrate assayed in the urine samples. Therefore, we filtered all serum samples to be assayed.

b) **Effect of hemolysis**: Blood samples from eight normal individuals were mechanically hemolyzed. The hemoglobin concentration in these samples ranged up to 12 g/L. These sera were analyzed concurrently with unhemolysed sera from the same subjects. The respective citrate values for the two groups were similar: 143.0 ± 20 and 144 ± 25 µmol/L (mean ± SD). We concluded that hemolysis does not affect the assay.

c) **Biochemical interferences**: Most of the transplant patients for whom we developed this method received cyclosporin A as an immunosuppressant, and blood cyclosporin concentrations range between 166 and 833 pmol/L. To a sample of each of the serum specimens from six normal subjects we added 833 pmol of cyclosporin A. These were filtered and analyzed simultaneously with the untreated sera. Cyclosporin A has a relative molecular mass of 1290 and is not retained by the filter. No differences were found in citrate results for the samples. Similarly, added lactate (up to 100 mmol/L) or pyruvate (up to 4 mmol/L) did not interfere with the assay. The normal reference interval for blood lactate in our laboratory is 0.7–1.8 mmol/L, for pyruvate 0.03–0.08 mmol/L.

We also studied the interference of icteric samples with citrate determination. Serum samples containing high bilirubin concentrations (up to five times the upper limit of normal, the upper limit in our laboratory for serum being 26 mmol/L) had similar concentrations of citrate up to 1400 µmol/L added and citrate determined. There were no differences between expected and observed values.

Analysis of Clinical Samples

We applied this method to determine citrate in liver transplant cases. The information listed in Table 2 is illustrative of the type of samples analyzed from one such case and the citrate values obtained. This has been made use of in obtaining citrate load and clearance data.

Discussion

This method is a rapid and apparently accurate procedure for analysis for citrate in blood and urine. Day-to-day and intra-assay precision of the assay is very good. The samples are stable for more than a month when stored at −20 °C. The assay reagents maintain stability when stored and handled as recommended by the manufacturer and can also be stored at −20 °C for four weeks after reconstitution without adverse effect on accuracy of analysis.

There are no analytical interferences from hemoglobin in venipuncture samples, from metabolites closely related to citrate such as lactate or pyruvate, from cyclosporin A, or from bilirubin.

The method shows linearity to 2700 µmol/L, sixfold the upper limit of the normal reference interval for blood (9). The samples can be diluted with water and the results correlate with the dilution factor. The method is suitable for either batch or single analysis and potentially could be automated. Per-sample cost of analysis is within the range for other frequently performed clinical analyses.

References


<table>
<thead>
<tr>
<th>Table 2. Citrate Concentrations in Clinical Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sample</strong></td>
</tr>
<tr>
<td>Plasma</td>
</tr>
<tr>
<td>Fresh frozen</td>
</tr>
<tr>
<td>From packed erythrocytes</td>
</tr>
<tr>
<td><strong>Patients’ samples</strong></td>
</tr>
<tr>
<td>Preoperative</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>During operation</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Postoperative</td>
</tr>
</tbody>
</table>

1222 CLINICAL CHEMISTRY, Vol. 30, No. 7, 1984
Evaluation of the Amniostat-FLM Assay for Assessment of Fetal Lung Maturity

Gillian Lockitch,1 Bernd K. Wittmann,2 Shirley M. Mura,1 and Louise C. Hawkley1

Results of the “Amniostat-FLM” assay, a rapid semiquantitative test for phosphatidylglycerol, were compared with determinations of the lecithin/sphingomyelin (L/S) ratio, with phosphatidylglycerol measured by two-dimensional thin-layer chromatography, and with results of the “shake test” for 94 specimens of amniotic fluid. Correlation between results with the Amniostat and the other tests was excellent. All four tests are very accurate when predicting lung maturity. The predictive value of a negative test, i.e., that hyaline membrane disease would not occur, was between 92 and 100%. However, the accuracy of predicted lung immaturity is poor. For all four tests, predictions of lung immaturity were incorrect in more than 50% of the cases. Of the 49 infants born within 72 h of testing, none developed hyaline membrane disease when phosphatidylglycerol was detectable by either method or when the shake test indicated fetal lung maturity, but three infants with L/S ratio >2/1 did develop the disease. The Amniostat provides a rapid screening method for detecting phosphatidylglycerol in amniotic fluid, which could well replace the thin-layer chromatographic method for measuring phosphatidylglycerol in the panel of diagnostic tests for fetal lung maturity.

Additional Keyphrases: fetal status • amniotic fluid • L/S ratio • screening • fetal lung maturity • “kit” methods

Reliable evaluation of fetal lung maturity and the ability to predict the respiratory distress syndrome caused by hyaline membrane disease (HMD) remain major diagnostic problems, particularly if active intervention in a pregnancy is being considered.3

Since 1971, when Gluck et al. (1) used thin-layer chromatography (TLC) to measure lecithin and sphingomyelin in amniotic fluid, this method has been the standard for assessing fetal lung maturity (2), an L/S ratio of 2/1 or greater generally indicating lung maturity. However, about 3% of all infants whose test results suggest lung maturity develop HMD (3). These are usually infants of diabetic or Rh-negative mothers. Moreover, some infants do not develop HMD even though born soon after a test has indicated lung immaturity; i.e., the proportion of false-positive results is substantial (4).

In addition, technical problems associated with the L/S ratio make interpretation of results difficult, i.e., if the amniotic fluid is contaminated with blood or meconium, has not been kept cold, or has been centrifuged under improper conditions (5). Because the TLC method requires about 2 h of technologist time and 3 h to provide a result, its use in many laboratories is restricted to regular weekday working hours.

In an attempt to increase the predictive value of these tests with amniotic fluid, the quantities of other phospholipids such as phosphatidylethanolamine, phosphatidylinositol, and phosphatidylglycerol (PG) were used to define a "lung profile" (3, 6). However, this further complicated the evaluation when it became obvious that various components of the lung profile could mature at different rates, producing confusion about which of these best reflected lung maturity (5).

Other methods proposed for determining fetal lung maturity are based on physical attributes of the amniotic fluid such as its microviscosity or surface tension—e.g., the "shake test" (7), which evaluates the fluid’s surface tension in terms of the stability of a ring of bubbles.

PG, which appears in amniotic fluid near the end of gestation, has been suggested as a more specific indicator of lung maturity than the L/S ratio (8). Because it is not found in the blood, this phospholipid can serve as an indicator of lung maturity even in blood-contaminated amniotic fluid (9).

Although it provides useful clinical data, the panel of tests

Received January 18, 1984; accepted April 9, 1984.