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Enzymic Determination of Citrate in Serum and Urine, with Use of the Worthington "Ultrafree" Device

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We describe an enzymic method for conveniently measuring citrate in serum or urine. Interfering enzyme proteins are removed by a disposable ultrafilter ("Ultrafree"; Worthington Diagnostics), obviating the need for hazardous protein precipitants. A 50 mmol/L Tris buffer adequately controls pH, no lactate dehydrogenase is necessary in the reagents, and the reaction of citrate catalyzed by citrate lyase (EC 4.1.3.6) is complete in 2–3 min. Within-run and day-to-day coefficients of variation were 7.5% and 5.4%, respectively. Serum citrate concentrations for 20 apparently healthy persons ranged from 0.08 to 0.17 mmol/L (mean 0.12, SD 0.03). Urinary citrate excretion by six normal volunteers ranged from 2.2 to 4.4 mmol/24 h. We observed no detectable changes in citrate in whole blood stored at room temperature for 90 min or longer. Overall, the method is faster and less hazardous than other methods for citrate that require protein precipitation.

Additional Keyphrases: *citrate (pro-3S) lyase · calculous disease · reactions to administration of bank blood · reference interval*

The deliberate excess of citrate in stored bank blood can cause symptoms in some recipients, which range from very mild to severe, although studies disagree as to the prevalence of these symptoms (*1*, *2*). The hypocalcemic effect of citrate is believed to be the cause of most of these symptoms, although the concentrations of potassium, phosphate, and lactate (e.g.) may also be important (*3*). In certain patients, measurement of citrate may be of use in documenting its assumed high concentrations and (or) rate of metabolism.

Studies of inhibition of calcium phosphate precipitation by citrate (*4*) and of decreased urinary excretion of citrate in persons with recurrent calcium urolithiasis (*5*, *6*) indicate that sufficient urinary citrate may be a factor in prophylaxis of renal stones. Reportedly, administration of bicarbonate to increase urinary citrate may help decrease the recurrence of renal stones (*7*). These observations could prompt requests for measurement of citrate in serum or urine, or both.

Several specific enzymic techniques have been published (*8–13*) for measuring citrate by using citrate (*pro-3S*) lyase (EC 4.1.3.6), which converts citrate to oxaloacetate and acetate. Malate dehydrogenase (EC 1.1.1.37) then converts the oxaloacetate to malate, with NADH consumed in the process. Moellering and Gruber (*8*) showed that this citrate lyase is highly specific for citrate. However, their preparation of citrate lyase contained a contaminant enzyme, oxaloacetate decarboxylase (EC 4.1.1.3), which required the addition of lactate dehydrogenase (LD; EC 1.1.1.27) to the reagents to offset this potential interference. In most methods published since then, LD is used (*6–8*, *10*). However, Toftegaard-Nielsen (*12*) described a commercially available citrate lyase preparation that is without such contamination and with which the use of LD is not necessary. He systematically determined optimal reagent concentrations, but the method requires protein precipitation, as have most (*8*, *10–12*), and also requires about 1 h to perform. Others found deproteinization unnecessary, but have either used suboptimal conditions (*9*) or have not given details of their procedure.[4]

Our modifications allow a more efficient use of reagents and time, combined with the recommended option of using a

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disposable ultrafiltration device (Worthington "Ultrafree"). Just why protein precipitation is needed has seldom been discussed; presumably it is to eliminate endogenous enzymes from the serum that might interfere by producing or consuming NADH. Our method requires only about 2 min for each analysis of ultrafiltrate, and the costly enzyme, citrate lyase, is more efficiently utilized. This manual technique requires no sample deproteinization, because the disposable ultrafilter eliminates enzymes that could change the NADH concentration. About 200 μL of serum is convenient for the assay, and LD need not be included in the reagents.

Materials and Methods

Equipment

Ultrafree disposable filters and autoinjector were obtained from Worthington Diagnostics, Freehold, NJ 07728. We used a Beckman Model 24 double-beam spectrophotometer here, but any spectrophotometer with which readings can be made at 340 nm may be used.

Stock Reagents

Tris(hydroxymethyl)methylamine (Tris) buffer, 100 mmol/L, pH 8.20 at 25 °C (Sigma Chemical Co., St. Louis, MO 63178).

NADH, disodium salt, supplied as a powder (cat. no. 107735) from Boehringer Mannheim, Indianapolis, IN 46250.

Malate dehydrogenase, about 6 MU/L, supplied in a solution of ammonium sulfate (Boehringer no. 127914).

$ZnSO_4$, 0.20 mmol/L.

Citrate lyase, 0.33 kU/g of powder (about 40 mg of the enzyme per gram of powder), supplied as a lyophilized powder (Boehringer no. 354074).

Citric acid stock standard, 10 mmol/L. Dissolve 192 mg of anhydrous citric acid in water and dilute to 100 mL.

Working Reagents

Reagent A. Mix 50 mL of Tris buffer, 50 mL of $ZnSO_4$, 7 mg of NADH, and 25 μL of malate dehydrogenase. Stored in a glass bottle at 4 °C, this mixture is stable for a month.

Citrate lyase, 14 kU/L (about 1.6 g/L). Dissolve 10 mg of the citrate lyase powder in 250 μL of Tris buffer. Store at 4 °C and use within 8 h. This amount suffices for about 20 tests.

Citric acid working standard. Dilute the stock standard with water to give a concentration of 500 μmol/L.

Quality-control material. Pool serum left over from routine analyses and mix it with 1 mL of 1 mol/L HCl per 100 mL of pooled serum. Filter the serum, then divide it into portions. Store at −20 °C. Thaw portions individually as needed. We found it necessary to prepare our own control material, because several commercial preparations we tested contained high concentrations of citrate.

Procedures

Serum preparation and analysis. Draw 200 to 400 μL of serum into a 1-mL plastic syringe, attach the Ultrafree filter and collect a little more than 100 μL of ultrafiltrate for the analysis. Mix 1.00 mL of Reagent A with 100 μL of ultrafiltrate and record the absorbance at 340 nm, with water as the blank. Add 10 μL of enzyme solution, mix thoroughly, and make a final absorbance reading when the reaction has stopped; this usually is within 2 min. Run the working standard and control as for patients and calculate sample results in millimoles per liter.

Procedure for urine. Dilute one volume of urine, collected with toluene as preservative, with 10 volumes of water, then mix 100 μL of this dilution directly with 1.00 mL of Reagent A. Record the initial absorbance at 340 nm. After adding 10 μL of enzyme, determine the net change in absorbance from the final absorbance reading.

With these concentrations of reagents and samples, the standard curve is linear to 0.7 mmol/L without dilution. The practical limiting factor in determining linearity is the NADH concentration.

Results

Concentration of citrate lyase. The high cost of citrate lyase encouraged us to determine the least amount necessary for a sufficiently fast reaction rate. We ran identical samples, using citrate lyase solutions with enzyme concentrations of 1.6, 2.4, and 3.2 g/L. The absorbance changes were the same, although the reaction took longer to complete as the concentration of enzyme decreased. A filtered sample containing 0.54 mmol of citrate per liter took about 2 min to complete, with use of 1.6 mg (14 U) of lyase per milliliter. The same sample took about 45 s with 27.2 U/mL. In contrast, an unfiltered sample containing 0.54 mmol/L citrate took 16 min to establish a curve that was suitable for determining the absorbance change.

The 1.6 mg/mL enzyme solution maintained an adequate reaction rate for 8 h; the 3.2 mg/mL solution could be used for 24 h. Because citrate assay would typically be offered only during some part of the working day, we used 1.6 mg/mL. This corresponds to a final enzyme concentration of 15 mg/L, or 140 U/L, in the mixture after addition to the sample and Reagent A.

Addition of LD to reagents. With 85 U of LD added to 100 mL of Reagent A, we saw no difference in the amount of absorbance change or the rate of the reaction in aqueous citrate standards, 0.1 to 0.5 mmol/L. Evidently, LD is not needed to compensate for impurities in the enzyme, as had been suggested (*8*).

We analyzed three sera for citrate, with and without LD in the Reagent A. Citrate concentrations in unfiltered serum, run with and without LD, differed by less than 0.01 mmol/L, and agreed with the values for filtered serum run with no LD. Ultrafiltrates analyzed with LD in Reagent A showed a nonlinear decrease in the initial absorbance before citrate lyase was added, which lasted 2–3 min. The ultrafiltrate probably contained pyruvate, which at a pH of 8.2 is rapidly converted by the additional LD to lactate, with a concurrent nonspecific decrease in the amount of NADH. With no LD added, the reaction with pyruvate proceeds at a negligible rate relative to that for the citrate. If endogenous LD and pyruvate are both very high, this might be noticeable as a drift in the initial absorbance readings. In such cases, more lyase should be added so that nonspecific absorbance changes are negligible during the reaction of citrate.

Effect of potassium. According to Toftegaard-Nielson (*12*), potassium increases the reaction rate. We found the opposite to be true: 400 mmol of KCl per liter, added to Reagent A, decreased the reaction rate.

Filtered vs unfiltered serum. Our initial studies had shown that none of the citrate in aqueous solution is adsorbed by the Ultrafree membrane, and that proteins such as amylase and albumin are essentially all retained (*13*). This provided an attractive alternative to the use of trichloracetic acid or perchloric acid as protein-precipitating agents. Continuous monitoring of unfiltered serum was required, because of nonspecific and unpredictable absorbance changes before and after addition of the citrate lyase, as shown in Figure 1. However, the absorbance change due to the conversion of citrate could be approximated by extrapolation. For ultrafiltrates the absorbance readings were stable before and after addition of citrate lyase; therefore, continuous scanning was not required. This saved time, and permitted the use of any

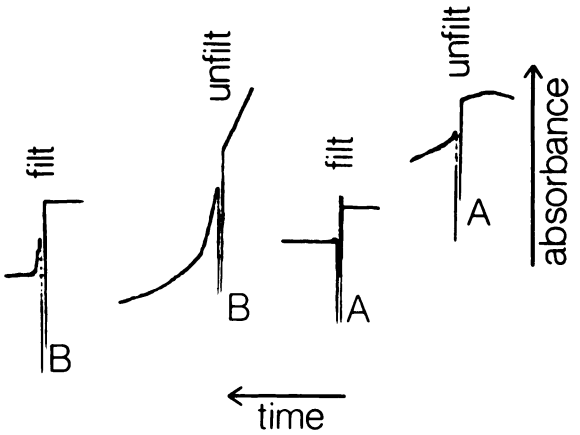

Fig. 1. Recording showing the elimination of nonspecific reactions by ultrafiltration of sera
A and *B* are different sera

spectrophotometer capable of measurements at 340 nm.

Fifty-four different sera and their corresponding protein-free ultrafiltrates were analyzed for citrate during a month. Two results were discarded because a value could not be obtained with the unfiltered samples. The citrate concentrations of the remaining 52 samples compared very favorably (r = 0.98). The regression equation was (filt.) = 1.00 (unfilt.) + 0.006 mmol/L.

Effect of processing time. To determine the effect of leaving serum on erythrocytes for various intervals at room temperature (22–25 °C), we drew five samples from each of three volunteers. Two samples contained heparin; one of these was centrifuged and analyzed immediately, the other after 60 min. Three sera were collected and centrifuged after 30, 60, and 90 min. Differences in citrate concentration did not exceed 20 μmol/L for any of the serum and plasma samples. We conclude that values for citrate are not detectably affected by clotting or the presence of erythrocytes, or both, for at least 90 min and that either serum or heparinized plasma can be used in this analysis.

Effect of commonly used urine preservatives. We added HCl to aqueous standards containing 0.2, 0.5, and 0.8 mmol of citrate per liter, to determine its effect on the citrate reaction. The final concentration of HCl was 60 mmol/L. It decreased the apparent citrate concentration by less than 20 μmol/L in all cases. Toluene, added to similar standards to a concentration of 3 mL/L, similarly altered the apparent citrate value by <20 μmol/L.

Reference interval. Sera was sampled from 20 apparently healthy volunteers and analyzed for citrate. The values ranged from 0.08 to 0.17 [mean 0.12 (SD 0.03) mmol/L]. This mean and range are consistent with those reported by Zender et al. (*9*) and Tomisek et al. (*11*). Citrate excretion by normal volunteers ranged from 2.2 to 4.4 mmol/24 h urine, values that agree with ranges reported by Schwille et al. (*5*) and Bach et al. (*6*).

Precision and analytical recoveries. A 20-day precision study was run on unfiltered pooled serum and on the ultrafiltrate obtained with the Ultrafree device. The unfiltered samples had a mean value of 0.12 mmol/L, an SD of 0.011, and a CV of 9%. The filtered samples had a mean value of 0.12 mmol/L, an SD of 0.0065, and a CV of 5.4%.

A within-run precision study, in which 15 different tubes of control sera were individually thawed, filtered, and analyzed, gave a mean value of 0.12 mmol/L, SD of 0.009, and CV of 7.5%.

We added 50 μL of the appropriate solution to 0.95 mL of both serum and urine so that citrate was in the increments stated in Table 1. Analyses of the serum ultrafiltrates and 10-fold dilutions of the urine indicated the recoveries to be quantitative.

Table 1. Analysis of Serum and Urine to Which Citrate Was Added

Sample + mmol/L added	Citrate, mmol/L
Serum	0.12
+0.1	0.23
+0.3	0.43
+0.5	0.63
Urine	1.45
+1.0	2.40
+2.0	3.22
+3.0	4.45

Linearity. With 70 mg of NADH per liter of reaction mixture, the standard curve for citrate was linear to 0.7 mmol/L. At 100 mg/L, linearity was extended but the initial absorbance reading was undesirably high.

Discussion

Our procedure is readily adaptable to routine clinical use. The Worthington Ultrafree filters provide a means of protein removal that is rapid and requires use of no hazardous chemicals. Tris is satisfactory for control of pH, because no neutralization of an acidic protein precipitant is necessary (*12*). The Tris concentration we used in our method adequately neutralizes the amount of acid that is commonly used in urine collections.

Protein removal is usually unnecessary in the case of urine, and occasionally unnecessary for serum. However, the endogenous enzymes in serum that consume or produce NADH usually make the analysis difficult or impossible without protein removal, as indicated in Figure 1.

Apparently the citrate lyase that is available commercially is without the contaminant enzyme oxaloacetate decarboxylase, which converts some of the oxaloacetate to pyruvate, without consumption of NADH (*8*, *11*), so LD is not needed in the reagent mixture. Furthermore, in whole blood from three healthy volunteers, we found no evidence that citrate is metabolized in vitro, because no changes were observed for at least 90 min after collection and storage of clotted blood at room temperature. This seems to be in agreement with Toftgaard-Nielsen (*12*) but contrary to the view of others (*9*, *11*). Urine requires refrigeration or a preservative (or both) during collection, because bacteria are likely to be present that may alter the citrate concentration.

Our method is suited for use in laboratories that require few such analyses. For those with greater needs, the method could probably be adapted to centrifugal analysis of the ultrafiltrates. The Worthington ultrafiltration devices seem to be a reliable and rapid alternative to protein precipitation. Other uses of this device could be in the analysis for galactose in serum (*14*) or fructose in seminal plasma (*15*), both of which require removal of protein. While most enzymic methods for oxalate in urine do not require protein removal (*16*), the measurement of oxalate in serum might require removal of interfering enzymes. For other applications, if the analyte of interest is partly bound to proteins, the values will be proportionately lower than the total concentration. Although citrate is bound to protein at pH 5.1, it reportedly is dialyzable at pH 7.4 (*17*). The clinical or physiological importance of free vs total levels of any particular constituent must also be considered, as with calcium (*13*).

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Effect of Sample Instability on Glycohemoglobin (HbA_1) Measured by Cation-Exchange Chromatography

Marcia Simon and J. David Hoover

We compared results for glycohemoglobin obtained from fresh whole blood or separated erythrocyte samples with results obtained after storage. We judged the storage condition to be acceptable ("stable") if the glycohemoglobin results after storage were within the 95 confidence interval (± SD) of the results obtained for the specimens on the day of venipuncture. Hemolysates can validly be stored for five months at −70 °C. Whole-blood samples stored at 4 °C remain stable for four days; whole blood treated with heparin or EDTA (but not oxalate) is stable for seven days. At 30 °C, whole blood or erythrocytes from some donors are stable for one day, but after two days and seven days, results are frequently higher. We confirmed previous findings that the separated erythrocytes can be stored at −20 °C for at least seven days. In addition, we compared the elution profiles for stored samples showing increased values.

Addition Keyphrases: *sample handling · variation, source of · preparation of control materials*

The assay for glycosylated hemoglobin (glycohemoglobin, HbA_1, fast Hb) is a new laboratory test with good potential for facilitating an improvement in the clinical management of patients with diabetes mellitus (*1–3*). Several techniques including colorimetry (*4*), isoelectric focusing (*5*), and RIA (*6*) have been described for glycohemoglobin quantitation, but more popular current methods are based on cation-exchange chromatography. The many published methods differ markedly in resolution and in suitability for the routine clinical laboratory (*7–9*). While some methods measure only HbA_{1c}, which is characterized by a covalent linkage of 1-amino-1-deoxyfructose to the N-terminal valines of the beta chains, most chromatographic methods measure the entire "fast" fraction, believed to include HbA_{1a} and HbA_{1b} (*7*). The source and function of the other minor hemoglobins are not yet fully understood (*2*).

The stability of the hemoglobins is fundamental to the preparation of quality-control materials for monitoring method performance and to the interpretation of results for patients' specimens after storage.

Few quantitative data have been published regarding the effects of sample instability on glycohemoglobin results. The present study was designed to evaluate storage conditions such as short-term delays encountered by specimens in reaching the laboratory and longer storage such as would be involved for quality-control materials used to monitor test performance and interlaboratory comparisons.

Materials and Methods

Glycohemoglobin assay. A description of the analytical method and its validation appears elsewhere (*7*). Briefly, we hemolyzed unwashed erythrocytes with carbon tetrachloride.

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