single Sep-Pak has been used for as many as 10 serum samples with no noticeable loss in efficiency.

For analysis of clinical samples, I compared the above sample pretreatment procedure to the acetonitrile extraction of Kabra et al. (1). The chromatographic conditions were similar to those of Kabra et al. (1), except for our use of ambient temperature and a 3.5 mL/min flow rate. The Sep-Pak pretreatment procedure resulted in samples that were significantly "cleaner" than the acetonitrile extracts when chromatographed. The height and duration of the extraneous peaks eluting near the solvent front were greatly reduced and the baseline was re-established before the anticonvulsants emerged. Hexobarbital can be used as an internal standard for this procedure.

This sample pretreatment procedure allows for reproducibility and sensitivity with less column degradation.

Reference

1. Kabra, P. M., Stafford, B. E., and Marton, L. J., Simultaneous measurement of phenobarbital, phenytoin, primidone, ethosuximide, and carbamazepine in serum by high pressure liquid chromatography. *Clin. Chem.* **23**, 1284–1288 (1977).

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An Enzymic Bicarbonate Reagent That Is Free of Pyruvate Interference

To the Editor:

Bicarbonate content of serum is determined enzymically by the use of the following reactions (*1–3*).

Phospho*enol*pyruvate + bicarbonate
$\xrightarrow[\text{carboxylase (EC 4.1.1.31)}]{\text{phospho}enol\text{pyruvate}}$ oxaloacetate + phosphate

Oxaloacetate + NADH + H^+
$\xrightarrow[\text{(EC 1.1.1.37)}]{\text{malate dehydrogenase}}$ malate + NAD^+

The NADH oxidation is followed at 340 nm and the bicarbonate content is calculated from either the change in absorbance for the overall reaction (*1, 2*) or from a rate measurement (*3*).

There are two major interferences with the second reaction. The principal one is endogenous pyruvate reduction, catalyzed by endogenous lactate dehydrogenase (EC 1.1.1.27). This source of error may be eliminated by a long preincubation to allow completion of the pyruvate reaction (*4*) or by including lactate dehydrogenase in the reagent. For an equilibrium method of measurement, these corrective measures require reading the initial absorbance at the end of the preincubation period and thus are not desirable, especially with automated analyzers.

A second possible cause of falsely high results may be enzymic (microorganisms) or nonenzymic decarboxylation of oxaloacetate to pyruvate (*5, 6*). The CO_2 formed in this reaction re-enters the reaction sequence and pyruvate reacts with NADH in the presence of lactate dehydrogenase, thereby oxidizing two equivalents of NADH for one oxalacetate. The extent of the decarboxylation can be expected to be negligible, because oxaloacetate is in a steady-state low concentration and the reaction is quickly completed. But adding exogeneous lactate dehydrogenase as suggested above would only accentuate this second interference.

An alternative solution, which obviates both sources of error, is to introduce an effective lactate dehydrogenase inhibitor. Of the reported inhibitors (*7–9*), oxamate is one of the most potent, forming a ternary enzyme–NADH–oxamate complex (*7, 10*). We have studied the inhibitory properties of oxamate and recommend its inclusion in an enzymic bicarbonate reagent.

Wheat phospho*enol*pyruvate carboxylase, pig-heart malate dehydrogenase, NADH, oxalacetate, and phospho*enol*pyruvate were obtained from Boehringer Mannheim, Indianapolis, IN 46250; tris(hydroxymethyl)aminomethane (Tris) and sodium oxamate from Sigma Chemical Co., St. Louis, MO 63178.

Phospho*enol*pyruvate carboxylase activity was determined by the method of Maruyama et al. (*11*).

Bicarbonate was assayed with a reagent, made in CO_2-free water, containing Tris buffer (pH 8.0, 50 mmol/L); NADH, 0.6 mmol/L; phospho*enol*pyruvate, 3.2 mmol/L; Mg^{2+}, 1.5 mmol/L; phospho*enol*pyruvate carboxylase, 500 U/L; malate dehydrogenase, 2500 U/L. After preincubating 1.6 mL of the reagent at 37 °C, 7.5 μL of sample was added and the net absorbance change was recorded at 340 nm. Equilibrium was achieved within 5 min.

We determined the activities of phospho*enol*pyruvate carboxylase and malate dehydrogenase in the presence of oxamate; concentrations up to 40 mmol/L had no effect on either enzyme.

When optimizing the oxamate concentration, we followed the lactate dehydrogenase/pyruvate reaction by omitting phospho*enol*pyruvate carboxylase and malate dehydrogenase from the bicarbonate reagent and adding oxamate. Since oxamate has been shown to be a competitive inhibitor with respect to pyruvate, its inhibitory properties were studied in the presence of the highest expected pyruvate concentration. Thus, we added 2 mmol of pyruvate per liter to human sera with normal and supranormal lactate dehydrogenase activities up to 2000 U/L. Also, in order to magnify the signal, the sample volume was increased from 7.5 to 50 μL. The oxidation of NADH was followed for 10 min at 340 nm and 37 °C, and quantitated. Without the inhibitor present, 10–40 μmol of NADH per liter was oxidized; when we added oxamate in increasing amounts, the oxidation was correspondingly reduced. At an oxamate concentration of 10–20 mmol/L, lactate dehydrogenase activity was completely inhibited.

Finally, we incorporated 10 mmol of oxamate in the reagent per liter, because a much smaller sample volume was used in routine bicarbonate determinations. We assayed samples with various lactate dehydrogenase activities, before and after adding 1 and 2 mmol of pyruvate per liter. The results showed no change in the bicarbonate concentration.

The lactate dehydrogenase/pyruvate reaction is the principal interfering NADH oxidant. This interference may be substantial, as shown in a recent report of a patient in septic shock whose specimen was found to have a pyruvate concentration of 1.4 mmol/L and a lactate dehydrogenase activity of 3000 U/L (*12*).

Because we used an equilibrium method, minor inhibitory effects of oxamate on the reagent enzymes are tolerable. However, any substantial inhibition would be of concern and might change the performance of the reagent. We found none on either enzyme.

References

1. Wilson, W., Jesyk, P., Rand, R., and Bevill, R. D., Use of the Vickers discrete analyzer for enzymatic determination of the bicarbonate content of serum. *Clin. Chem.* **19**, 640 (1973).

2. Forrester, R. L., Wataji, L. J., Silverman, D. A., and Pierre, K. J., Enzymatic method for determination of CO_2 in serum. *Clin. Chem.* **22**, 243–245 (1976).

3. Menson, R. C., Narayanswamy, V., Bussian, R. W., and Adams, T. H., A kinetic method for determination of CO_2 in biological fluids. *Clin. Chem.* **20**, 872 (1974).

4. Liese, W., Jung, K., Ludtke, B., and Grützmann, K. D., Kommentar zur Standardvorschift des DAB 7 (DL)-DDR. Bestimmung der Activität der Aspartataminotransferase in Serum. *Zentralbl. Pharm.* **114**, 1051–1064 (1975).

5. Bergmeyer, H. U., Scheibe, P., and Wahlefeld, A. W., Optimization of methods for aspartate aminotransferase and alanine aminotransferase. *Clin. Chem.* **24**, 58–73 (1978).

6. Rej, R., and Vanderlinde, R. E., Assay of aspartate aminotransferase activity: Effects of serum and serum proteins on oxaloacetate

decarboxylation and dialysis. *Clin. Chem.* **20,** 454–464 (1974).

7. Novoa, W. B., Winer, A. D., Glaid, A. J. and Schwert, G. W., Lactic dehydrogenase V. Inhibition by oxamate and by oxalate. *J. Biol. Chem.* **234,** 1143–1148 (1959).

8. Emerson, P. M., and Wilkinson, J. H., Urea and oxalate inhibition of serum lactate dehydrogenase. *J. Clin. Pathol.* **18,** 803–807 (1965).

9. Martinek, R. G., A rapid ultraviolet spectrophotometric lactic dehydrogenase assay. *Clin. Chim. Acta* **40,** 91–99 (1972).

10. Winer, A. D., Novoa, W. G., and Schwert, G. W., Observation of new phenomena in the fluorescence spectrum of a diphosphopyridine nucleotide-linked dehydrogenase. *J. Am. Chem. Soc.* **79,** 6571–6572 (1957).

11. Maruyama, H., Easterday, R. L., Chang, H. C., and Lane, M. D., The enzymatic carboxylation of phospho*enol*pyruvate. *J. Biol. Chem.* **241,** 2405–2412 (1966).

12. Hanson, N. Q., and Yasmineh, W. G., The effect of pyruvate on NAD^+/NADH coupled enzyme reaction systems. *Clin. Chem.* **25,** 1159 (1979).

13. Plummer, D. T., and Wilkinson, J. H., Organ specificity and lactate dehydrogenase activity. *Biochem. J.* **87,** 423–429 (1963).

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Improved β-Zone Resolution in Agarose Gel Electrophoresis

To the Editor:

In serum protein electrophoresis, increased resolution in the β zone (transferrin, C3 complement, β-lipoprotein) may be important in the diagnosis of certain abnormalities (*1*). For example, the acute-phase response is characterized in part by increased C3 complement and decreased transferrin (Tf) concentration (*1, 2*). With inadequate Tf/C3 separation, these opposing effects may not be seen. As an indicator of total iron-binding capacity, Tf concentration may be useful in the diagnosis of iron-deficiency anemia (*2*). Decreased C3 is useful in diagnosis of autoimmune diseases such as systemic lupus erythematosus (*1, 3*). Finally, the ability to resolve the β-lipoprotein should permit nephrotic syndrome and protein-losing enteropathy to be distinguished (*4*).

We have adapted a commercially available, pre-cast, thin-layer agarose gel electrophoresis system for improved serum protein separation, particularly in the β zone. The modified buffer was prepared by dissolving one vial of "Universal" buffer (Corning Medical and Scientific, Palo Alto, CA 94306; cat. no. 470180) and 870 mg of calcium lactate (Sigma Chemical Co., St. Louis, MO 63178; cat. no. L-2000) in about 1 L of de-ionized water. When dissolution was complete, the pH was adjusted to 8.6 and the volume brought to 1.5 L instead of 2.0 L as suggested by the supplier. The better resolution in the β zone results from the presence of calcium lactate in both the gel and the running buffer. The buffer in the agarose gel, which does not contain calcium lactate, must therefore be replaced. To do this, we place a single sheet of "Sta-Moist" (Corning, cat. no. 470158) paper on the gel ("Universal" gel, Corning, cat. no. 470100) surface for 15 s, then soak the gel for at least 10 min in the modified "Universal" buffer described above. After draining the gel for 1 min, the sample wells were blotted with a soft, lint-free tissue (Kimwipe) and 1 μL of serum was applied to each sample well. The two chambers of the cell base (Corning) were filled with 75 mL of the modified "Universal" buffer, and electrophoresis performed for 30 min at 125 V. (At 90 V, 45 min is required for equivalent separation.) Proteins were simultaneously fixed and stained for 10 min with a 5 g/L solution of Amido Black 10B in dilute (50 mL/L) acetic acid. Excess stain was removed by rinsing the gel in dilute acetic acid. The gel was then dried for 20 min, cleared by soaking in two successive 200-mL dilute acetic acid baths for 2 min each, and dried again (this requires about 10 min). The dry, cleared gel is examined visually and densitometrically. The entire procedure takes approximately 90 min.

Fig. 1. Serum protein electrophoresis pattern by the modified agarose gel method described here

With Amido Black 10B stain, fractions consist mainly of the following: *1*, albumin; *2*, α_1-antitrypsin; *3*, α-2 macroglobulin and haptoglobin; *4*, transferrin; *5*, C3 complement; *6*, β-lipoprotein; and *7*, IgG, IgM, and IgA

As shown in Figure 1, seven distinct protein zones are seen on the resulting patterns, as compared to five previously reported (*5*). The additional two bands migrate as β_2 and β_3 globulins. In order of decreasing electrophoretic mobility, the three β bands have been identified as transferrin, C3 complement, and β-lipoprotein. Identification was achieved by (*a*) adding purified transferrin (Sigma, cat. no. T-2252) to normal samples, (*b*) precipitating C3 complement with rabbit antiserum to human C3c (Calbiochem-Behring Corp., San Diego, CA 92112, cat. no. 527301), and (*c*) running samples known to have above-normal β-liproprotein concentrations (>99th percentile). The location of β-lipoprotein was confirmed by applying the same samples to both halves of a gel, staining half of the gel with Amido Black 10B (protein stain), the other half with Fat Red 7B (lipid stain), and superimposing the two halves. The Tf and C3 bands were further examined by correlation with total iron-binding capacity (Sigma cat. no. 565) and by radial immunodiffusion (Helena Laboratories, Beaumont, TX 77704; cat. no. 9324), respectively.

We find this modified serum protein procedure to be faster than a proposed Selected Method (*6*), and it does not require the use of picric acid, which may be hazardous (*7*).

References

1. Killingsworth, L. M., Cooney, S. K., and Tyllia, M. K., Protein analysis: The closer you look, the more you see. *Diagn. Med.* **3,** 47–59 (1980).

2. Alper, C. A., Plasma protein measurements as a diagnostic aid. *N. Engl. J. Med.* **291,** 287–290 (1974).

3. Laboratory Notes: Serum Proteins No. 3, November 1973. Behring Diagnostics, Somerville, NJ.

4. Sun, T., Lien, Y. Y., and Gross, S., Clinical application of a high resolution electrophoresis system. *Ann. Clin. Lab. Sci.* **8,** 219–227 (1978).

5. Rosenfeld, L., Serum protein electrophoresis: A comparison of the use of thin-layer agarose gel and cellulose acetate. *Am. J. Clin. Pathol.* **62,** 702–706 (1974).

6. Jeppsson, J. O., Laurell, C. B., Franzen, B., et al., Agarose gel electrophoresis. *Clin. Chem.* **25,** 629–638 (1979). Proposed Selected Method.

7. *Clin. Chem.* **26,** 804 (1980).

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Postcoital Enzyme Activities in the Vagina

To the Editor:

Any work that advances the ability of the forensic scientist to investigate sexual offenses is to be welcomed, as such offenses by their very nature invariably pose particularly difficult problems for our courts of law. Any new published work on this matter therefore deserves the closest attention. We refer to the work of Gohara (*1*). In our opinion, by failing to take into account the work of earlier researchers, Gohara oversimplifies the true state of affairs, both with respect to the identification of semen and also to the conclusion that may be drawn from studying the rate of decay of enzymes in the vagina following coitus. Our reasons are as follows: