Rapid-Flow Enthalpimetric Determination of Urea in Serum, with Use of an Immobilized Urease Reactor

Larry D. Bowers, Leslie M. Canning, Jr., Curt N. Sayers, and Peter W. Carr

We used a differential thermal detector in conjunction with an immobilized urease reactor to determine urea in serum. Samples (120 μl) are introduced into a flow stream and passed through an "adiabatic" column, which is packed with enough insolubilized urease to completely convert urea to ammonia and carbon dioxide. Measured temperature changes are directly proportional to the serum urea concentration. Urea in the presence of protein, bilirubin, and hemoglobin can thus be rapidly, simply, and inexpensively measured. Results correlate well with those obtained by the manual diacetyl monoxime and urease/indophenol methods.

Additional Keyphrases: immobilized enzyme • inter-method comparison • thermoanalytical methods

Determination of urea is the most widely used index to protein catabolism and renal function. Methods for its determination have most often been based on spectrophotometric or fluorometric measurements after directly coupling urea to diacetyl monoxime type compounds (1-3) or on the urease-catalyzed hydrolysis of urea to ammonia and carbon dioxide (4-6). Most of these methods require either a multienzyme system, possibly handicapped by kinetic problems, or may be invalidated by adventitious contamination with ammonia. Here, we report a technique that does not rely on determination of ammonia or carbon dioxide, and that utilizes the catalytic selectivity of urease (EC 3.5.1.5).

Lately, interest in the field of clinical applications of enthalpimetric techniques has increased significantly (7-11). Because almost all chemical reactions are accompanied by the production or absorption of heat, enthalpimetric methods provide a universal reaction detector, the chief problem of which is its universality—i.e., its sensitivity to heat generated by undesired reactions. This difficulty can be overcome by coupling the thermal sensor to a highly specific enzyme reaction. Enthalpimetric techniques may be subject to two additional problems: a lack of sensitivity, which necessitates large sample volumes when low concentrations are to be measured, and long thermal equilibration phenomena, which lower the rate of analysis. We have attempted to develop a new type of flow enthalpimetric analyzer, which incorporates the well-known advantages of an immobilized enzyme reactor. The basic principles of enthalpimetric analysis have been well detailed elsewhere (8, 10, 12).

Materials and Methods

Reagents

Enzymes and reagents for urea determination. A reagent kit based on the diacetyl monoxime condensation reaction was obtained commercially (Harleco “Blood Urea Nitrogen Reagents and Standards Kit,” cat. no. 635-6, lot no. 5031G, supplied by A. H. Thomas Co., Philadelphia, Pa. 19105). A second set of reagents was prepared according to the Chaney-Marbach modification (5) of the urease/indophenol determination. A 100-mg portion of urease (Type IV; Sigma Chemical Co., St. Louis, Mo. 63178) was added to 1.0 g of disodium ethylenediaminetetraacetate (EDTA) and diluted to 100.0 ml with phosphate buffer (0.1 mol/liter, pH 7.0). The phenol color reagent and alkaline hypochlorite solution were prepared as described by Henry (13). All
solutions were refrigerated until required. The urease solution was always used within one week of its preparation.

Standard urea solutions. Urea stock standards were prepared by dissolving 1.0717 g (17.9 mmol) of urea (Baker Analyzed, J.T. Baker Co., Phillipsburg, N.J. 08865) in 100.0 ml of a sodium chloride/EDTA solution (9 g/liter and 20 mmol/liter, respectively). The working standards were prepared by appropriate dilution of the stock standard with the same diluent as above. We also prepared a set of standards in a protein matrix by dissolving the requisite amount of urea in a bovine serum albumin solution (70 g/liter) that also contained sodium chloride (9 g/liter) and EDTA (20 mmol/liter). These solutions were refrigerated when not in use.

Apparatus

The apparatus is diagrammed in Figure 1. The major components of the system are a well-stirred, thermally unregulated Dewar-type bath (from a Model 4000 Heat of Adsorption Detector; Varian Aerograph, Walnut Creek, Calif. 94598), a differential thermistor system (Model GB41M2; Fenwal, Framingham, Mass.), and an "adiabatic" column, which is packed with urease immobilized on controlled porosity glass (see below). The columns were insulated units with the inside tube (5 mm i.d., 2.5 cm long, and 0.6 cm wall thickness) surrounded by a glass jacket. The region between the two walls was evacuated but not silvered (University of Georgia Glass Shop, Athens, Ga. 30601). We found that these columns could be operated at a flow rate of 1 ml/min with complete conversion of considerable quantities of urea (11). The temperature change, as indicated by the thermistors, was monitored on a strip-chart recorder as the disbalance voltage of a differential ac phase-lock bridge (11, 14). A Model 801 pH meter (Orion, Cambridge, Mass. 02139) was used for all pH measurements. All spectrophotometric measurements were made with a Spectronic 88 spectrophotometer (Bausch & Lomb, Rochester, N.Y. 14625).

Preparation of Covalently-bound Urease

Urease was immobilized on controlled porosity glass (CPG, 50 nm pore diameter, 200–400 mesh; Electronenetics, Springfield, N.J. 07086) by means of a bifunctional silane reagent and glutaraldehyde cross-linking (15–17).

A 50 ml/liter solution of glutaraldehyde was prepared by adding 20 ml of glutaraldehyde (250 ml/liter solution, Grade II; Sigma Chemical Co.) to 80 ml of phosphate buffer (0.1 mol/liter, pH 7.0) and placed over 1.0 g of controlled porosity glass, which had been silanized with γ-aminopropyl-triethoxysilane (Pierce Chemical Co., Rockford, Ill. 61105) (18). The 25-ml Erlenmeyer flask containing the reaction mixture was connected to an aspirator and maintained at reduced pressure for 1 h. During this period, air bubbles were removed from the pores in the glass and the glass acquired a yellow hue that darkened with increasing reaction time. After an additional 2 h of reaction at atmospheric pressure, the glass was washed five times with buffer, five times with sodium chloride (0.5 mol/liter), and five additional times with buffer. Urease (Type IV, Sigma) was dissolved in the above phosphate buffer to give a concentration of 100 mg/ml and 1 ml of this solution was added to the moist glass. After 5 min of additional de-gassing of the pores, the vessel was placed in a refrigerator for 18 h. The glass was washed as above and stored as a wet cake in the refrigerator until used. The final product was brick red.

Analysis Procedure

A stock solution of sodium chloride and EDTA (9 g/liter and 20 mmol/liter, respectively) was prepared and adjusted to pH 7.4 with solid potassium hydroxide. One liter of phosphate buffer (2 mol/liter) was prepared by dissolving 60.5 g KH2PO4 and 271.0 g K2HPO4 in the stock sodium chloride/EDTA solution. A solution of 100.0 ml of buffer and 300.0 ml of stock NaCl/EDTA solution was used as the pump reservoir solution. The pump (Model RRPS4; Fluid Metering Inc., Oyster Bay, N.Y. 11771) was turned on and after 5 min (for equilibration) the disbalance of the Wheatstone bridge was nulled.

The samples were prepared by mixing three parts of serum or standard with one part of the phosphate buffer solution (2 mol/liter) in a 5-ml disposable beaker. After swirling to mix the components, the sample-buffer was drawn into the sample loop (120 μl) of a sampling valve (Model SV8031; Chromatronix, Berkeley, Calif. 94710) and introduced into the flow stream. The temperature peak resulting from the enthalpy of conversion of urea to ammonia and carbon dioxide in the "adiabatic" column is displayed on a strip-chart recorder. A typical
example of the observed enthalpogram (temperature/time curve) is shown in Figure 2. The data of Figure 3 indicate that a linear calibration curve can be obtained for urea nitrogen concentrations from 5 to 70 mg/100 ml. The peak height was measured as the maximum deflection from the pre-peak baseline.

**Results and Discussion**

One of the drawbacks of thermoanalytical methodologies in the past has been the inability of the technique to differentiate between heats from various sources. In the present method, for example, the glass surface of the support matrix and (or) the bound enzyme could act as nonspecific ion-exchange sites, yielding heat from the ion-exchange process. As reflected in the analysis procedure, this requires regulation of the ionic species present (19). We were concerned about the possibility of the protein present in the serum matrix taking part in the ion-exchange process. As can be seen from Figure 3, the response to both protein-containing and protein-free matrices is nearly identical.

The precision and accuracy of the results obtained by the analysis of commercial quality-control sera (Versatol Kit, lot No. 2670064, and Calibrate Set, lot No. 271084) are shown in Table 1. As a measure of accuracy, the enthalpimetric technique was compared to several common methods. As can be seen, the thermal method agrees quite well with both the diacetyl monoxide (1) and urease/indophenol (5) methods. The precision of the present method is also acceptable. The measurement precision may be limited by the system noise, which is only evident when the scale is expanded to measure very low concentrations, or by the sample injection process. For the data of Table 1, the precision is limited by the injection valve. An increase in precision may be obtainable with the use of peak integration techniques. We attempted to use analog integration to measure the peak area, but baseline shifts after the peaks made quantitation difficult. Digital integration may facilitate improvements in precision. The day-to-day repeatability of the method over an 18-day period was 9% for a 0.1 mmol/liter urea solution.

Recovery and interference studies (Table 2) were

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**Table 1. Results (in Terms of Urea N) of Analysis of Commercial Quality Control Sera for Urea Nitrogen by Three Methods**

<table>
<thead>
<tr>
<th>Quality-control sera</th>
<th>Stated value</th>
<th>Method</th>
<th>Urea nitrogen, mg/100 ml</th>
<th>CV, % ( n = 5 )*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Versatol</td>
<td>13.3</td>
<td>Thermal</td>
<td>12.6</td>
<td>0.48</td>
</tr>
<tr>
<td>Versatol A</td>
<td>29.4</td>
<td>Urease</td>
<td>28.8</td>
<td>0.26</td>
</tr>
<tr>
<td>Versatol Alternate</td>
<td>61.2</td>
<td>Diacetyl</td>
<td>55.0</td>
<td>0.30</td>
</tr>
<tr>
<td>Calibrate 1</td>
<td>10</td>
<td>Indophenol</td>
<td>10.3</td>
<td>0.50</td>
</tr>
<tr>
<td>Calibrate 2</td>
<td>41</td>
<td></td>
<td>40.0</td>
<td>0.59</td>
</tr>
<tr>
<td>Calibrate 3</td>
<td>80</td>
<td></td>
<td>74.0</td>
<td>0.56</td>
</tr>
</tbody>
</table>

* For enthalpimetric method, with five replicates of each quality-control serum. Samples run randomly within a single day.
performed with a specimen of pooled sera obtained from the University Health Service. The pool was dialyzed against a sodium chloride/tris(hydroxymethyl)aminomethane buffer (9 g/liter and 0.1 mol/liter, pH 7.4, respectively) with use of a fiber dialyzer (Dow Fiber Dialyzer, Model 6/HFD-1). Known amounts of urea and interferents were weighed into volumetric flasks and dissolved in the dialyzed protein matrix. A second approach involved addition of a known amount of urea to an undialyzed pool. Results of the enthalpimetric technique agree very well with those by other methods.

Because urease is a very specific enzyme, interferences are expected to be minimal. The major objection to other urease-based techniques has been the detection of adventitious ammonia either from analytical reagents or the air. As can be seen from Table 2, ammonia does not interfere with the present technique. In addition, bilirubin and hemoglobin, which are common spectrophotometric interferences, do not influence the thermal technique in any way.

Comparison studies of the results obtained for fresh sera by the present method and the manual diacetyl monoxime and urease/indophenol methods are shown in Figures 4 and 5. The individual samples were obtained from Athens General Hospital and frozen until used. The three procedures were then run as nearly simultaneously as possible. The correlation between the enthalpimetric technique and the urease/indophenol

Table 2. Recovery and Interference Studies for Urea in Sera

<table>
<thead>
<tr>
<th>Initial concn, mg/100 ml</th>
<th>Amount added, mg</th>
<th>Concentration detected</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Thermal</td>
</tr>
<tr>
<td>0</td>
<td>2.5</td>
<td>2.6</td>
</tr>
<tr>
<td>0</td>
<td>40</td>
<td>39.6</td>
</tr>
<tr>
<td>32</td>
<td>38</td>
<td>72.0</td>
</tr>
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</table>

Interference study

<table>
<thead>
<tr>
<th>Interferent</th>
<th>Concen, mg/100 ml</th>
<th>Concen of urea, mg/100 ml</th>
<th>Apparent urea concentration, mg/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄Cl</td>
<td>15</td>
<td>40</td>
<td>Thermal: 39.5, Urease/Indophenol: 56, Diacetyl monoxime: 41</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>750</td>
<td>40</td>
<td>Thermal: 39.7, Urease/Indophenol: 42, Diacetyl monoxime: 43</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>10</td>
<td>40</td>
<td>Thermal: 39.7, Urease/Indophenol: 39, Diacetyl monoxime: 44</td>
</tr>
</tbody>
</table>

* Data are in terms of amount of urea nitrogen per 100 ml of dialyzed serum.

Fig. 4. Correlation of enthalpimetric urea method with urease/indophenol (Chaney–Marbach) procedure

Results: slope, 0.984 ± 0.030; Intercept, 0.159 ± 0.693 (mg/dl); correlation coefficient, 0.986; Student’s t-value, 32.8. All concentrations are mg of urea nitrogen per 100 ml

Fig. 5. Correlation of enthalpimetric method with the diacetyl monoxime (Fearn) procedure

Results: slope, 0.978 ± 0.056; Intercept, 0.48 ± 0.11 (mg/dl); correlation coefficient, 0.972; Student’s t-test, 18.5. All concentrations are mg of urea nitrogen per 100 ml
method is excellent because both methods rely on the urease-catalyzed hydrolysis of urea for quantitation. Correlation with the manual diacetyl monoxime method is not as good. Because of the extreme precision required for the reaction temperature and heating time and the photosensitivity of the color, the scatter observed was probably due to the colorimetric procedure rather than the thermochemical method.

The enthalpimetric method possesses a number of other significant advantages. First, it requires only the preparation of a buffer solution, and no dangerous or noxious materials are used for color development. Second, sample preparation requires only two pipettings. Third, the use of an immobilized urease column allows the rapid use of an equilibrium or end-point technique. Not only is the method capable of high analysis rate (~40/h), but also is simple enough to allow both emergency and routine urea analyses to be performed with identical conditions. The linear range of the technique is determined primarily by the amount of immobilized enzyme in the column. In the system reported here, linearity extends to at least 75 mg of urea nitrogen per liter. With a larger column, we were able to achieve linearity to over 5.0 g/liter. An additional advantage is inherent in the stability of the enzyme column, which has functioned for over a month under conditions of daily use.

Thermochemical methodologies have been conspicuously absent from the clinical laboratory, because they tend to be complex, insensitive, and time-consuming. In contrast, flow enthalpimetric techniques such as that described here combine the universality of thermal techniques with the advantages and specificity of an immobilized enzyme to provide a useful, speedy analytical tool. Conditions such as the presence of protein, hemolysis, or lipids are not important.

Numerous other applications for this technique are feasible and investigations are continuing in this laboratory.

We thank Dr. Carl A. Burtis of Oak Ridge National Laboratory for the loan of the Varian bath, and the National Institutes of Health for a grant (GM 17913) for support of this work. One of us (LDB) wishes to express his gratitude to the Graduate School of the University of Georgia for a graduate fellowship.

References