Ultramicrodetermination of Plasma Urea by Reaction with Diacetylmonoxime–Antipyrine without Deproteinization

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Urea can be directly determined in serum or plasma by reaction with diacetylmonoxime–antipyrine: 5 μl of the sample is mixed with 5 ml of the chromogenic reagent, and the mixture is boiled 15 min, cooled, and read at 460 nm. The color is stable for 30 min in the light. The reagent is composed of (*a*) 0.4 g of antipyrine per 100 ml of H_2SO_4 (20 ml/100 ml), containing 5 mg of ferric sulfate per 100 ml: 1 (or 2) volume(s), and (*b*) 0.5 g of diacetylmonoxime per 100 ml of acetic acid (5 ml/100 ml): 1 volume.

Additional Keyphrases *AutoAnalyzer*

Avoidance of a deproteinization step, especially in widely used reactions, is an obvious advantage in manual, discrete, or continuous-flow automated procedures. One way to avoid it is to use dilution instead of precipitation or dialysis. This necessitates very sensitive and specific methods of determination, completely free from secondary reactions with proteins, and non-opalescence, which can persist at low plasma or serum concentrations.

These possible causes of error must be completely eliminated, within large safety limits, to overcome quantitative and qualitative differences in protein composition among samples.

The method for urea determination based on the diacetylmonoxime–antipyrine reaction was introduced a few years ago (*1*, *2*). Owing to its very high sensitivity and specificity, it seemed particularly suited to the dilution procedure. In fact, a sample-to-reagent ratio of 1 to 1000 can be used. However, as described later, in working with the original reagent, even at this dilution, a brownish discoloration and, especially with plasma, a faint opalescence is seen. High sulfuric acid concentration and long boiling time may account for these inconveniences.

A search for a modified, satisfactory reagent and procedure was undertaken, and the results are reported here.

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Received Nov. 23, 1970; accepted Feb. 4, 1971.

Materials and Methods

Reagents

Solutions for the chromogenic reaction:

(*a*) H_2SO_4 (20 ml/100 ml) containing 4 g of antipyrine (2,3-dimethyl-1-phenyl-3-pyrazolin-5-one; Bayer) and 50 mg of $Fe_2(SO_4)_3 \cdot 9\ H_2O$ per liter.

(*b*) Diacetylmonoxime, 0.5 g per 100 ml of acetic acid (5 ml/100 ml).

These solutions are indefinitely stable at room temperature.

Reagent I: 8 parts of *a* are mixed with 1 part of *b*.

Reagent II: 2 parts of *a* are mixed with 1 part of *b*.

Reagent III: 1 part of *a* is mixed with 1 part of *b*.

These reagents must be used immediately after their preparation. Reagent I is the originally described reagent, used with deproteinized solutions.

The solutions used for deproteinizing were:

Soln A—Borate buffer, 0.1M, pH 11.0:

Boric acid, 6.2 g, is dissolved in 150 ml of 1N NaOH and diluted to 1 liter with water.

Soln B—Cadmium sulfate solution:

$CdSO_4 \cdot 8\ H_2O$, 102 g, is dissolved in 240 ml of 1N H_2SO_4 and diluted to 1 liter with water.

Procedure

Recommended procedure. Reagents II or III can be used interchangeably. To 5 ml of reagent, 5 μl of plasma is added by an especially designed

micropipet (*5*), mixed on a mechanical shaker, or by glove-covered hands, and boiled for 15 min. Direct contact with the fingers must be avoided, because of possible contaminations by urea from sweat.

After boiling, the tubes are cooled under running water and read at 460 nm against a blank made by the boiled reagent.

A standard, 1 g of urea per liter, is run with every series of determinations.

$$\text{Calculation: } \frac{A \text{ unknown}}{A \text{ standard}} = \text{g of urea per liter}$$

Absorptivity is high: 5,400. Although this is also very convenient for accurate determinations of small amounts of urea, it may not be so for high concentrations. In these cases dilution of the color with the reagent allows a correct reading. I observed that when samples containing urea at 10 g/liter concentration were subjected to the reaction, and the color formed was diluted 10-fold, the same absorbance was obtained as with a 1 g/liter solution.

Evaluation and Results

Evaluation of the interference of proteins with the various reagents. Urea was destroyed by incubating serum for 30 min at 37 °C with an equal volume of a soluble urease solution (Merck; 5 U/mg, 30 °C), 1 mg/ml in 0.067M phosphate buffer, pH 7.0. After incubation, 10 µl (corresponding to 5 µl of serum) was added to 5 ml of reagents I, II, and III; the mixtures were boiled for 25 min in a water bath, then cooled and read at various wavelengths against their respective blanks. Absorbance was observed between 400 and 520 nm when reagent I was used, but there was none with reagents II and III (Figure 1). Although not exceptionally great, this absorbance could introduce important errors in the presence of low concentrations of urea.

Influence of proteins on urea determination. To evaluate the interference of the color produced by proteins with reagent I on urea determination in untreated plasma and sera, I performed parallel determinations with all three reagents directly, without deproteinization and for reagent I, also on a deproteinized filtrate of the same material. Deproteinization was by adsorption on cadmium hydroxide (*3*, *4*).

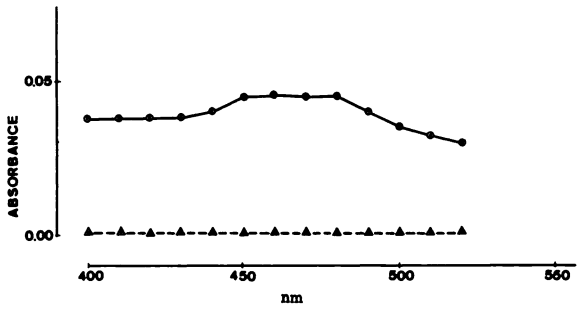

Fig. 1. Influence of plasma proteins on the diacetylmonoxime-antipyrine reaction for urea determination, with various reagents

Urea was destroyed by a preliminary treatment of plasma by urease. Some brown color and opalescence develop with reagent I, ⊙—⊙. No influence is observed with reagents II and III, △- - -△

To 6.6 ml of deproteinizing solution *a* 0.4 ml of plasma or serum was added, mixed, and 1 ml of solution *b* was introduced. The precipitate formed was centrifuged or filtered away after 10 min; 0.1 ml of filtrate (corresponding to 5 µl of plasma or serum) was used for the reaction with 5 ml of reagent. The reactions with the various reagents were performed under their optimal conditions as subsequently specified. As Table 1 shows, the determinations with reagents II and III gave results indistinguishable from those obtained on the deproteinized filtrate; in contrast, the results with reagent I without deproteinization were excessively high. The error is especially evident with plasma, and as expected its influence is particularly important at low urea concentrations.

Rate of color appearance and its stability. Owing to the interference of proteins on reagent I it was used only on deproteinized filtrates to compare it with reagents II and III in regard to the influence

Table 1. Diacetylmonoxime-antipyrine Reaction on Deproteinized Filtrates and Directly on Sera and Plasmas with Various Urea Concentrations, Using Reagents I, II, or III

Deproteinized filtrate	Not deproteinized					
	Serum			Plasma		
Reagent I	Reagent I	Reagent II	Reagent III	Reagent I	Reagent II	Reagent III
	Absorbance at 460 nm					
0.110	0.135	0.112	0.108	0.165	0.110	0.105
0.125	0.155	0.125	0.122	0.175	0.125	0.120
0.140	0.172	0.145	0.140	0.200	0.140	0.135
0.240	0.268	0.245	0.238	0.295	0.240	0.235
0.250	0.280	0.250	0.250	0.300	0.245	0.250
0.255	0.288	0.255	0.250	0.310	0.250	0.250
0.520	0.555	0.530	0.525	0.615	0.595	0.580
0.570	0.600	0.570	0.565	0.625	0.565	0.570
0.600	0.625	0.605	0.595	0.650	0.600	0.595

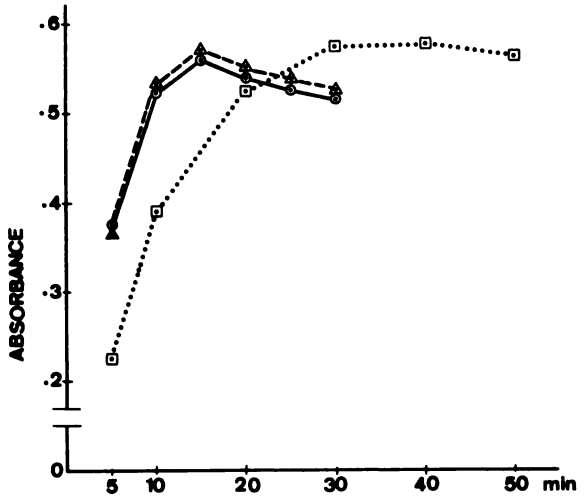

Fig. 2. Color developed after various boiling times in the presence of reagents I ⊡.....⊡, II ▵- - -▵, and III ⊙—⊙

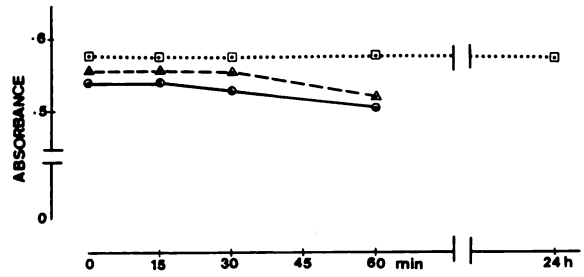

Fig. 3. Stability of the color produced by reagent I (25 min boiling) ⊡.....⊡, II ▵- - -▵, and III ⊙—⊙ (15 min boiling)

of boiling time on the rate of appearance of color and on its stability.

Plasma or sera, 5 μl, and 0.1 ml of filtrate, respectively, were added to 5 ml of reagent, boiled for various times and then read at 460 nm *vs.* the respective blanks, immediately after cooling and at several times thereafter. The results are summarized in Figure 2.

With reagent I the maximum color is developed after 25 min; with the other reagents the color develops more rapidly, and is complete in 15 min. Further boiling results in a color decrease. The duration of boiling, together with the reagent composition, influences the stability of the color. That produced by reagent I during 25–30 min of boiling is stable for longer than 24 h; that produced by reagents II and III boiling for 15 min is stable for about 30 min and then slowly decreases (Figure 3). With these reagents, 67% of the maximum color develops in 5 min of boiling, and is as stable as the color produced by a 15-min boiling.

Intercomparison of the three reagents and an automated procedure. A series of 250 plasma specimens was analyzed with reagents II and III, using 5 μl of plasma and 15 min of boiling, and with reagent I with a corresponding amount of deproteinized filtrate and 25 min of boiling. At the same time the determination was performed on the AutoAnalyzer, with the diacetylmonoxime–thiosemicarbazide method. The same results were obtained by all the methods used, with and without deproteinization. Also in comparison with the AutoAnalyzer a correlation of 1.0 (±0.7%) was determined. Hemolysis or high bilirubin content did not interfere.

Recovery experiments. A recovery of 100.0% ± 1.5% was observed for 10 plasma samples.

Reproducibility. On 20 successive determinations on the same plasma having a urea content of 0.42 g/liter a standard deviation of ±2.5% was obtained.

Discussion

With a convenient dilution of the sulfuric acid in the chromogenic reagent, interference by proteins can be completely avoided and urea determined on untreated plasma. The method seems to be easily adaptable to both discrete and continuous-flow analysis. Application to flow analysis is under study in our laboratory.

References

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