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IMPROVEMENTS IN THE DETERMINATION OF UREA USING DIACETYL MONOXIME; METHODS WITH AND WITHOUT DEPROTEINISATION

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Summary

A rapid and reproducible method is described for measurement of urea in biological materials (after deproteinisation) and in serum (without deproteinisation). Urea is colorimetrically determined with diacetyl monoxime and thiosemicarbazide in the presence of sulphuric acid, phosphoric acid and ferric chloride. The sensitivity of the colorimetric reaction and stability of the colour are enhanced over existing related procedures and the serum blank diminished, enabling urea to be precisely measured in micro amounts (1—5 μl) of serum.

Introduction

There were many problems with the original Fearon [1] method, including reactions between constituents of the chromogenic reagent, prolonged boiling time, instability of the colour to light and time, low sensitivity, and a non-linear calibration curve, suggesting more than one chromogenic reaction. Various improvements have been made over the years, but a systematic re-investigation of reaction conditions has now eliminated these shortcomings almost completely.

We have found also that non-ionic detergents diminish the blank absorbance due to serum proteins to such an extent that an assay can be run on 1—5 μl serum, without deproteinisation, and with accuracy sufficient for routine purposes.

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Recommended methods

A. Measurement of urea with deproteinisation

Reagents

(1) Acid-ferric solution. Add 100 ml concentrated phosphoric acid (85%, d = 1.67, Mallinckrodt Co., St. Louis, MO, U.S.A.) to 300 ml of concentrated sulphuric acid (95—98%, d = 1.84, E. Merck) and 600 ml distilled water. Dissolve 100 mg ferric chloride in the above solution. For the purposes of this paper the volume of this and similar solutions is taken to be 1 litre.

(2) Diacetylmonoxime (DAMO)-thiosemicarbazide (TSC) solution. Dissolve 500 mg DAMO (Sigma Chemical Co.) and 10 mg TSC (Sigma) in distilled water and dilute to 100 ml.

(3) Chromogenic reagent. Mix two parts of Reagent 1 with one part of Reagent 2 immediately before use.

Procedure

To 0.1 ml of deproteinised sample add 3 ml of chromogenic reagent (Reagent 3). Mix vigorously and boil in a water bath for 5 min. Cool to room temperature and read absorbance at 525 nm against a blank composed of distilled water and chromogenic reagent. The amount of urea present can be obtained from a standard curve (0—150 nmol urea). In the present work all absorbances were measured with a Varian Series 634 double beam spectrophotometer.

For deproteinisation, we have used either trichloroacetic acid (TCA) or perchloric acid (PCA) (5% final concentration) followed by removal of precipitate by centrifugation.

B. Measurement of urea in serum without deproteinisation

Reagents

Prepare acid-ferric solution, diacetylmonoxime-thiosemicarbazide solution, and chromogenic reagent as described above.

Procedure

To 5 μl of serum add 3 ml of chromogenic reagent followed by 0.1 ml of Brij-35 solution (Sigma Chemical Co.). For the most accurate work, it may be worth also preparing serum blanks of each sample using serum pre-treated with urease (below). Mix vigorously and heat for 5 min in a boiling water bath. Cool and measure absorbance at 525 nm against a blank composed of 5 μl of distilled water plus 3 ml of reagent 3 and 0.1 ml of Brij-35 solution. A standard curve is prepared with varying concentrations of urea (0—150 nmol) in a final volume of 5 μl of distilled water.

Serum blanks may be prepared for each sample by adding 10 μl of urease solution (Type VII, Sigma Chemical Co., 0.1 mg in 1 ml distilled water) to 10 μl of serum, incubating for 5 min at 37°C and then carrying 5 μl of the resultant mixture through the procedure detailed above. The correct blank absorbance for use in calculation is obtained by multiplication with the dilution factor (X2).

For ordinary purposes correct for serum blank by subtracting 0.4 nmol (0.08 mmol/l when using 5 μl serum) from the result read off from the standard curve.

Results and discussion

After extensive preliminary search, the starting-point selected for optimisation was the method of Coulombe and Favreau [2] which uses DAMO in phosphoric acid, and with thiosemicarbazide as colour stabiliser. Our final conditions give a sensitivity similar to that achieved by Ceriotti and Spandrio [3—5], who used DAMO with acetic and sulphuric acids, Fe^{3+} to accelerate the reaction, and antipyrine which both accelerates the reaction and modifies the final colour. In the present method the heating time is shorter, the colour more stable, and the serum blank less.

Table I compares reaction conditions in these and a number of other recent methods based on the same principle, and includes some detail on performance.

Establishment of optimum conditions

Acid reagent composition

Coulombe and Favreau [2] noted that sulphuric acid gave higher colour

TABLE I

A COMPARISON BETWEEN PRESENT AND SEVERAL PREVIOUS METHODS FOR ANALYSIS OF UREA

Ref. No.	Constituents of chromogenic reagent		Heating time (min)	Colour stability in fluorescent light (h)	Final volume (ml)	Absorbance for 100 nmol urea
	acids	other reactants				
(2)	H_3PO_4	DAMO TSC	20	2	5.2	0.090 *
(4)	H_2SO_4 CH_3COOH $Fe_2(SO_4)_3$	DAMO antipyrine	40	<0.5 *	10	0.370 *
(5)	H_2SO_4 CH_3COOH $Fe_2(SO_4)_3$	DAMO antipyrine	15	<0.5 *	5.1	0.743 *
(6)	H_2SO_4 HCl CH_3COOH $MnCl_2$ NaH_2PO_4 $NaNO_3$	DAMO phenyl-anthranilic acid	11—12	0.5 **	6.2	≃0.360 **
Present method	H_2SO_4 H_3PO_4 $FeCl_3$	DAMO TSC	5	2	3.1	0.940

* As found by us.

** As reported in the original.

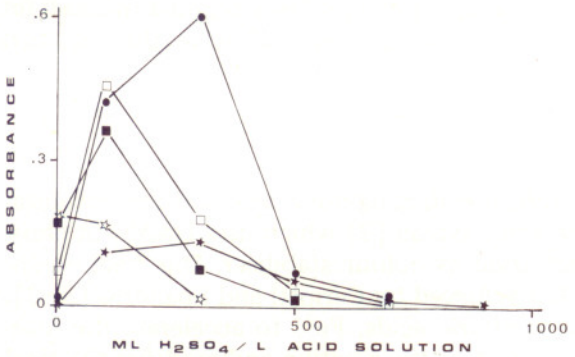

Fig. 1. Effect of sulphuric acid and phosphoric acid concentrations on colour formation. 0.1 ml of urea solution (1 mmol/l) was boiled for 5 min with 1 ml DAMO-TSC solution (5 g DAMO and 50 mg TSC per litre) and 2 ml acid solution containing varying concentrations (ml/l) of H_2SO_4, (i) in absence of H_3PO_4 (★———★); and in presence of (ii) 100 ml H_3PO_4/l (●———●); (iii) 300 ml H_3PO_4/l (□———□); 500 ml H_3PO_4/l (■———■); and (iv) 700 ml H_3PO_4/l acid solution (☆———☆).

yields than phosphoric acid, but chose the latter for simplicity in preparing the reagents. Fig. 1 shows that mixtures of these two acids give better colour yield than either alone, with an optimum in the region of 100 ml and 300 ml per litre respectively of concentrated phosphoric and sulphuric acids.

Concentration of ferric, manganous and chloride ions

Catalytic acceleration by ferric salts was reported by Ceriotti and Spandrio [4] and there is a report [6] of sensitisation by Cl^- and colour stabilisation by Mn^{2+}. We found maximum colour development with 100 mg $FeCl_3$ per litre of acid solution, slightly higher absorbances with the chloride than the sulphate, and some depression of absorbance above the optimum. Manganous chloride depressed colour production at all concentrations tested.

Concentration of thiosemicarbazide

Thiosemicarbazide is essential for stability of colour. We experimented with increasing concentrations and found a fairly sharp optimum at 100 μg per assay tube. Above this there was an increase in blank absorbance.

Concentration of diacetylmonoxime

A sharp increase in absorbance was found up to 5 mg per assay tube. Thereafter there was no further change up to 10 mg per assay tube.

Heating cycle

Using the recommended reagents and procedure, absorbance reached its maximum within 5 min in a boiling water bath and thereafter decreased slightly with time. The water bath was covered to minimise evaporation and to exclude light.

Deproteinising agents

Solutions of urea were prepared in trichloroacetic acid (0—450 g/l) and perchloric acid (0—540 g/l). 0.1-ml lots were then tested by the recommended procedure. There was no detectable variation in absorbance.

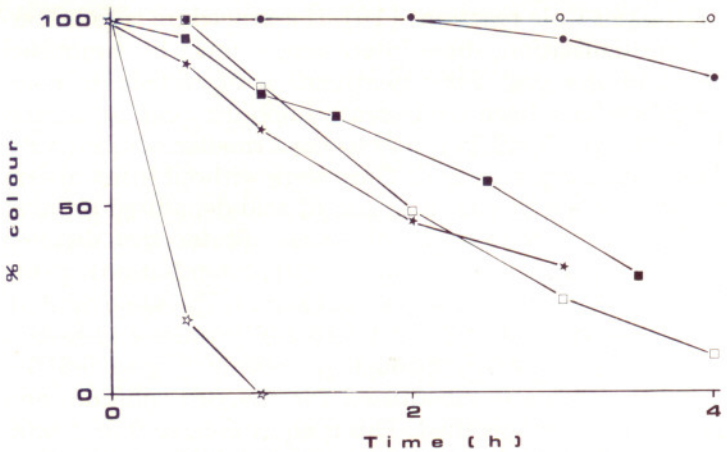

Fig. 2. Effect of light on colour stability. 0.1 ml of urea solution (1 mmol/l) was boiled for 5 min using optimum reagent and conditions as described under "Recommended Methods". After boiling, the assay tubes were kept for 4 h in (i) moderate light (○———○); (ii) intense illumination with fluorescent light (●———●); and (iii) sunlight (□———□). Results are expressed as absorbance at different time periods as a percentage of absorbance at zero time, i.e. immediately after boiling. In similar experiments with Ceriotti's methods, colour was developed with 1 mg urea and the tubes then exposed under fluorescent light as for (ii) above. ☆———☆, 5 min boiling; ★———★, 40 min boiling (method as ref. 4). ■———■, 15 min boiling (method as ref. 5).

Spectrum and linearity

The colour produced by serum samples showed an absorbance spectrum indistinguishable from that given by pure urea solutions (λ_{max} 525 nm). The standard curve was linear up to the highest concentration tested (150 nmol urea) and the colour yield from 100 nmol urea was 0.940 absorbance units (525 nm) (in 3.1 ml final volume).

Stability of colour (Fig. 2)

When exposed to sunlight, colour was stable for 0.5 h, after which there was apparently an autocatalytic acceleration of fading. Under artificial light from fluorescent tubes the colour showed little change up to 2 h after heating. When kept in the dark or moderate illumination colour was stable for more than 5 h. Under similar conditions of fluorescent light exposure, the colour developed by Ceriotti's methods [4,5] began to fade within 0.5 h. Moreover, the proportionate rate of fading was dependent on urea concentration, more colour being lost with lesser urea concentrations (10 nmol) than with higher (100 nmol and above) concentrations. These observations may indicate a cooperative effect of light, reaction intermediates and breakdown products of the chromophore. The better stability achieved in our procedure is then presumably attributable to approximate completion of the chromogenic reaction.

Procedure without deproteinisation

In the presence of strong mineral acids serum proteins may give rise to brown pigments [5,7,8] or to turbidity and precipitation [9]. Probably there is also a contribution to blank absorbance from carbohydrates in the serum which are dehydrated to furfural derivatives and then react with tryptophan present

in the serum proteins (Hopkins-Cole reaction) [10]. If a procedure is to be carried through without deproteinisation, these interferences must be minimised and the approaches used in the past for determination of carbamido compounds like urea or citrulline have been to decrease sulphuric acid concentration (but with loss of sensitivity [5,8,9,11], and to use a smaller sample [5,8]. We found non-ionic detergents very effective. They were without influence on the colour reaction and perhaps act by solubilisation of acid-denatured protein.

Thirty normal serum samples were treated with urease, diluted with distilled water and carried through procedure A (but omitting deproteinisation), giving an absorbance of 0.024 ± 0.003 (mean ± S.D.) for 5 μl serum. The same diluted, urea-free samples gave absorbances of 0.017 ± 0.010 (460 nm) by Ceriotti's procedure [5]. The same samples carried through procedure B gave 0.010 ± 0.002 with 10 g Lubrox-WX (Sigma)/l substituted for the Brij solution, and 0.004 ± 0.003 with the Brij solution specified. This is equivalent to 0.08 ± 0.06 mmol urea/l serum and can either be ignored, or corrected for by subtracting uniformly 0.08 mmol/l from the result, or corrected for by applying individual serum blanks. For 25 serum samples with assay results in the normal range, the greatest proportionate error introduced by ignoring the correction was 3.4%.

5 μl was chosen as the sample size purely as a matter of convenience and in view of the difficulty of accurate measurement of smaller volumes. The assay is capable of measuring the urea present in 1—2 μl of normal serum, when the blank becomes quite negligible. The uniform correction suggested above is valid for a 5-μl sample.

Reproducibility, etc.

Procedure B gave a coefficient of variation of 3.02%, calculated from 15 determinations on a single sample divided between two batches. A comparison with Ceriotti's non-deproteinisation procedure [5] showed good agreement and confirmed the much diminished serum blank correction required in the present method.

Interferences

Several other compounds produce colour complexes with diacetyl monoxime but are of little importance when considering serum because present in negligible amount or yielding little colour (allantoin) [12]. The citrulline content of serum is usually too low to interfere, but the raised levels of argino-succinate synthetase deficiency (citrullinaemia — 14 cases known up to 1978 [13]) would give a significant non-urea response.

Advantages

The method is sensitive, simple and quick — especially simple when using the method without deproteinisation and even then is amply accurate and reproducible for routine work. It should be easily adapted for automation. Further, the colour produced is more stable to light than previously reported and the reagents are simple to prepare and stable on storage.

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References

1 Fearon, W.R. (1939) Biochem. J. 33, 902—907
2 Coulombe, J.J. and Favreau, L. (1963) Clin. Chem. 9, 102—108
3 Ceriotti, G. and Spandrio, L. (1963) Clin. Chim. Acta 8, 295—299
4 Ceriotti, G. and Spandrio, L. (1965) Clin. Chim. Acta 11, 519—522
5 Ceriotti, G. (1971) Clin. Chem. 17, 400—402
6 Beale, R.N. and Croft, D. (1961) J. Clin. Pathol. 14, 418—424
7 Siest, G. and Vigneron, C. (1968) Clin. Chim. Acta 20, 373—379
8 Ceriotti, G. (1973) Clin. Chim. Acta 47, 97—105
9 Vassef, A.A. (1978) Clin. Chem. 24, 101—107
10 Holmes, E.J. (1968) J. Histochem. Cytochem. 16, 136—146
11 Ohsita, M., Takeda, H., Kamiyama, Y., Ozawa, K. and Honjo, I. (1969) Clin. Chim. Acta 67, 145—152
12 Mclean, P., Novello, F. and Gurney, N.W. (1965) Biochem. J. 94, 422—426
13 Shih, V.E. (1978) in The Metabolic Basis of Inherited Disease, 4th Ed. (Stanbury, J.B., Wyngaarden, J.B. and Fredrickson, S., Eds.), pp. 371—386, McGraw-Hill, New York