Technical methods

Manual and automated methods for measuring urea based on a modification of its reaction with diacetyl monoxime and thiosemicarbazide

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Since the reaction between urea and diacetyl monoxime was first described (Fearon, 1939) it has formed the basis of most laboratory methods of estimating urea. Many modifications of the technique as originally described have since been suggested in order to try and overcome some of the difficulties of the strongly acid solutions required, light sensitivity of the coloured product of the reaction, and the deviation of the reaction from Beers Law. The advent of automatic systems of analysis and in particular of the AutoAnalyzer¹ has reduced the severity of some of these problems but has by no means eliminated them.

The method until recently advocated by the Technicon Company, and widely used in Britain, is basically that of Marsh, Fingerhut, and Kirsch (1957) but includes the modified ferric alum acid reagent suggested by Richter and Lapointe (1962). This method utilizes an acid reagent consisting of 33% v/v sulphuric acid and 33% v/v phosphoric acid, and a diacetyl monoxime reagent in 15% w/v sodium chloride. These reagents are expensive to produce and dangerous to use. A further complication is that there is no simple manual procedure based on this reaction which is available for use in emergency determinations. Hence laboratories tend to use the urease method for emergency work resulting in two entirely different chemical principles being used for one determination which one would very much wish to avoid.

Recently further modifications of the basic method have been suggested by the introduction of other reagents into the reaction. In Australia Beale and Croft (1961) used phenylanthranilic acid while in the United States thiosemicarbazide and phenazone have proved popular (Ceriotti and Spandrio, 1963; Coulombe and Favreau, 1963; Moore and Sax, 1965).

In this paper the thiosemicarbazide method of Marsh, Fingerhut, and Miller (1965) has been modified to provide automated and manual methods suitable for use in a routine laboratory.

In the automated method the 10 mm colorimeter flow cell has been replaced by a 15 mm tubular flow cell and one of the air lines has been omitted. These modifica-

tions have enabled the sample pump tubing to be reduced in size from 0.035 in. ID to 0.025 in. ID while still achieving an increase in sensitivity sufficient to warrant dilution of all specimens containing urea in excess of 150 mg/100 ml which was considered desirable.

In the manual procedure concentrations of reagents have been adjusted to give maximum colour development without developing the turbidity which is found if an excess of DAM or TSC is used. The water bath time has also been reduced from the 20 minutes of Marsh *et al* to five minutes.

In order to achieve optimum colour development it has proved necessary to increase the volume of fluid taken following protein precipitation from 0.2 to 0.5 ml.

REAGENTS

AUTOMATED PROCEDURE

- 1 Stock diacetyl monoxime (DAM) 2.5% w/v in water
- 2 Stock thiosemicarbazide (TSC) 0.25% w/v in water
- 3 5% w/v anhydrous ferric chloride in 1% w/v sulphuric acid
- 4 Working DAM/TSC reagent, 200 ml stock TSC, and 400 ml stock DAM made to 2,000 ml with water
- 5 Acid reagent 1,000 ml water, 80 ml concentrated sulphuric acid, 10 ml 85% phosphoric acid, and 10 ml 5% ferric chloride
- 6 0.9% sodium chloride solution
- 7 Standards of 10, 25, 50, 100, and 150 mg/100 ml urea. These standards were made up in a solution containing 0.2 g phenyl mercuric acetate and 1.4 ml concentrated sulphuric acid in 5 litres distilled water.

MANUAL PROCEDURE

- 1 Stock reagents as for the automated procedure
- 2 Acid reagent as above
- 3 10% trichloroacetic acid (TCA)
- 4 Working DAM/TSC 24 ml stock DAM and 10 ml stock TSC made to 100 ml
- 5 Colour reagent 25 ml acid reagent and 5 ml DAM/TSC prepared freshly before use
- 6 Stock standard of 100 mg urea/100 ml as above
- 7 Working standard 10 ml stock with 50 ml 10% TCA to 100 ml with water
- 8 Blank solution 50 ml 10% TCA made to 100 ml with water.

METHODS

AUTOMATED The AutoAnalyzer system shown in Fig. 1 proved to be satisfactory. Samples can be run at 60 per hour. A specimen tracing is shown in Figure 2. All specimens containing urea in excess of 150 mg/100 ml are diluted with saline and repeated.

MANUAL To 0.8 ml distilled water in a centrifuge tube add 0.2 ml of blood, plasma, or serum followed by 1 ml of 10% TCA. Mix well and centrifuge.

¹Technicon Instruments Company, Chauncey, N.Y.
Received for publication 19 December 1967.

FIG. 1. Diagram of the AutoAnalyzer system used in these investigations.

FIG. 2. A typical AutoAnalyzer tracing obtained by the method described showing:

- A Peaks obtained from a series of standards
- B The effect of contamination of one specimen by another
- C Continuous aspiration of one specimen
- D Repeated aspirations of one solution
- E A series of peaks obtained with 5 sera chosen at random.

Take 0.5 ml supernatant into a test tube. Into another tube place 0.5 ml of standard solution and into another 0.5 ml of blank solution. To each tube add 5 ml of colour reagent, mix, and immerse in a boiling water bath for five minutes. Remove, cool, and read against the blank at 520 mμ or with a green filter.

$$\text{Calculation} = \frac{\text{Test}}{\text{Standard}} \times 100 = \text{urea (mg/100 ml)}$$

All results in excess of 150 mg/100 ml should be repeated by diluting the supernatant $\times 5$ before treatment with colour reagent.

Urine can be estimated by both manual and automated procedures but should first be diluted 1 to 20 with water.

NOTES

In the manual method a water bath time of five minutes has been selected since it is long enough to develop sufficient colour for accuracy and ease of measurement while being short enough for the entire estimation to be completed within 15 minutes. It is not essential that this heating time be very strictly adhered to but prolonged heating must be avoided or destruction of the coloured product will occur (Fig. 3).

The DAM/TSC reagents for both manual and automated techniques develop a yellow colour within a few days of being made up. This does not affect their usefulness. The acid reagent is stable indefinitely but the DAM/TSC reagent for the manual method very slowly decreases in potency and it is advisable to replace each batch at monthly intervals.

The anticoagulants in common laboratory use, i.e. heparin, EDTA, fluoride, and oxalate have all been

FIG. 3. Tracing of colour intensity against heating bath time for specimens put through for manual procedure described. The time for maximum colour production is nine minutes but a time of five minutes has been chosen for this work and proved to be satisfactory.

investigated and shown not to interfere with the reaction.

Interference filters of 520 $m\mu$ have been used in this investigation but 505 and 550 $m\mu$ filters have also been shown to be satisfactory.

An absorption curve of the red complex produced in this reaction is shown in Figure 4.

RESULTS

Fifty-two specimens of plasma with urea concentrations between 14 and 181 mg/100 ml were chosen at random and investigated on the AutoAnalyzer both by the reported method and by the standard Technicon method. The mean difference between the two methods was shown to be -1.7 mg/100 ml, the standard deviation of the difference between pairs being 2.2 mg/100 ml and the greatest difference 6 mg/100 ml.

Forty-five specimens with urea concentrations varying between 17 and 189 mg/100 ml previously determined

on the AutoAnalyzer were estimated by the manual procedure. The mean difference between pairs was -0.16 mg/100 ml with a standard deviation of the difference between pairs of 3.8 mg/100 ml, the greatest individual difference being 9 mg/100 ml.

A standard graph is shown in Fig. 5 made up of urea solutions varying from 10 to 200 mg/100 ml investigated by the manual procedure. It will be seen that the curve bears a close relationship to linearity over the range 0 to 150 mg/100 ml while beyond this the deviation from linearity is considerable. Hence an upper limit of 150 mg/100 ml was chosen for the manual assay and specimens having ureas in excess of 150 mg/100 ml were required to be diluted.

FIG. 5. Graph obtained when a series of standards was put through the manual procedure. Note the close approximation to linearity over the range 0 to 150 mg/100 ml, and the divergence from linearity of specimens having ureas in excess of 150 mg/100 ml.

SUMMARY

Manual and automated techniques are described for the determination of urea in body fluids by means of a modification of the diacetyl monoxime thiosemicarbazide procedure. It is suggested that since the methods are chemically similar and incorporate only low concentrations of caustic reagents they might usefully replace other methods used in routine hospital laboratories.

I wish to thank Dr E. F. Bowers, Mrs J. A. Highfield, and Mrs E. M. Jukes for their assistance.

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FIG. 4. Absorption curve shown by the red complex produced in the described reaction.