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Automated Determination of Urea Nitrogen in Serum with Diacetyl Monoxime-Glucuronolactone Reagent (Organic Analysis. LXXIII[1])

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New automated methods are presented for the determination of urea nitrogen in serum using undiluted and diluted samples. It is based on the previously established color reaction of urea with diacetyl monoxime in phosphoric acid solution in the presence of D-glucuronolactone. The methods give reproducible and reliable results, and the diacetyl monoxime-glucuronolactone reagents modified for the methods are very stable.

Several methods have been reported for the automated determination of urea nitrogen in biological fluids, which based on the direct reaction of urea with diacetyl monoxime in an acidic medium. The currently recommended Technicon N-1b method[3] is a modification of the Skeggs and Marsh procedures.[4,5] The original reaction of the method required corrosive sulfuric acid and gave a sigmoidal calibration curve which caused a poor sensitivity at a lower concentration of urea nitrogen. In order to eliminate the latter disadvantage, a known amount of urea was added to the diacetyl monoxime reagent in Technicon N-1b method. But this compromized treatment made the reagent solution unstable. Recently, the diacetyl monoxime reaction was improved by adding thiosemicarbazide to the reagent solution to obtain an adequate sensitivity,[6] and the new procedure has been automated for the determination of urea nitrogen in blood and urine.[7,8] However, the diacetyl monoxime-thiosemicarbazide reagent was unstable, and an excessive amount of thiosemicarbazide interfered the color development and caused turbidity in the reaction mixture.[7]

In a previous paper[9a], D-glucuronolactone was found to produce a stable color when added to the reactoin of urea and diacetyl monoxime in phosphoric acid solution, eliminating sigmoidal nature of calibration curve in the determination. This reaction was successfully applied to the determination of urea nitrogen in blood and urine with a simple procedure, and then extended for the microdetermination in blood.[9b] This paper describes an adaptation of the manual method to an automated determination of urea nitrogen in serum, and presents two procedures which use undiluted and diluted samples (direct serum method and diluted serum method).

1) Part LXXII: Y. Ohkura, J. Murakami, J. Shiota and T. Momose, *Chem. Pharm. Bull.* (Tokyo), **18**, 2164 (1970).
2) Location: *Katakasu Fukuoka.*
3) "Technicon Autoanalyzer Method File N-1b," Technicon Instruments Corporation, Chauncy, New York.
4) L.T. Skeggs, *Am. J. Clin. Path.*, **28**, 311 (1957).
5) W.H. Marsh, B. Fingerhut and E. Kirsch, *Am. J. Clin. Path.*, **28**, 681 (1957).
6) J.J. Coulombe and L. Favreau, *Clin. Chem.*, **9**, 102 (1963).
7) W.H. Marsh, B. Fingerhut and H. Muller, *Clin. Chem.*, **11**, 624 (1965).
8) S. Joseph and B.S. Annino, *Am. J. Clin. Path.*, **48**, 147 (1967).
9) *a*) T. Momose, Y. Ohkura and J. Tomita, *Clin. Chem.*, **11**, 113 (1965); *b*) T. Momose, Y. Ohkura and T. Imaizumi, *Yakugaku Zasshi*, **86**, 678 (1966).

Experimental

Reagents[10]

Reagents for the Direct Serum Method——Potassium Chloride Solution: Dissolve 9 g of KCl and 0.5 ml of 20% Brij-35[11] in H_2O and dilute to 1000 ml.

Diacetyl Monoxime-glucuronolactone Reagent: Dissolve 20 g of diacetyl monoxime, 10 g of D-glucuronolactone,[12] 50 g of KCl and 0.5 ml of 20% Brij-35 in H_2O and dilute to 1000 ml. This solution is stable for at least 1 year when stored in a cool place under the protection from light.

Phosphoric Acid Solution: Dilute 400 ml of H_3PO_4 (85%) and 0.5 ml of 20% Brij-35 with H_2O to 1000 ml.

Phenylmercuric Acetate Solution: Dissolve 0.4 g of phenylmercuric acetate in about 400 ml of H_2O by warming. After cooling, add 2.8 ml of conc. H_2SO_4 and dilute with H_2O to 1000 ml. This solution is used for the preparation of urea nitrogen standard solutions as preservative and diluent.

Urea Nitrogen Standard Solutions: Dissolve 2.1433 g of dried urea in the phenylmercuric acetate solution and make up to 1000 ml. The solution contains 100 mg/dl urea nitrogen. Using this solution, prepare dilutions corresponding to 5, 10, 20, 30, 40 and 50 mg/dl urea nitrogen with the diluent. The solutions are stable for at least 1 month when stored in a refrigerator.

Reagents for the Diluted Serum Method——Sodium Chloride Solution: Dissolve 9 g of NaCl and 0.5 ml of 20% Brij-35 in H_2O and dilute to 1000 ml.

Diacetyl Monoxime-glucuronolactone Reagent: Dissolve 5 g of diacetyl monoxime, 5 g of D-glucuronolactone, 150 g of NaCl and 0.5 ml of 20% Brij-35 in H_2O and dilute to 1000 ml. This solution is stable when stored in the same way as the corresponding reagent in the direct serum method.

Phosphoric Acid Solution: Dilute 800 ml of H_3PO_4 (85%) and 0.5 ml of 20% Brij-35 with H_2O to 1000 ml.

Urea Nitrogen Standard Solutions: Prepare first 10 mg/dl urea nitrogen solution by diluting the 100 mg/dl solution described before with the phenylmercuric acetate solution. Using this solution, prepare dilutions corresponding to 1, 2, 3, 4, 5, 6, 7 and 8 mg/dl urea nitrogen with the diluent. The solutions are stable for 1 month when stored in a refrigerator.

Instrument——Basic type auto-analyzer[13] was used. Sampler II module, a dialyzer equipped with type C membrane and a tubular 15 mm flow cell colorimeter equipped with 480 mμ interference filters were employed.

Direcet Serum Method——Flow Diagram and Procedure: The flow diagram is shown in Fig. 1a. About 0.5 ml of serum placed in a sample cup is aspirated into the system at the rate of 40 samples/hr and diluted with KCl solution. The mixture is dialyzed against diacetyl monoxime-glucuronolactone reagent. The dialysate is mixed with H_3PO_4 solution and heated in a heating bath of 95° equipped with single heating coil. The transmittance of the resulting yellow orange mixture is measured while the mixture passed through the flow cell in the colorimeter, and recorded as Chart Division. The value of urea nitrogen (mg/dl) is read on the calibration curve, prepared as described below. If the value exceeds 50 mg/dl, it is necessary to repeat the treatment using 2 times-H_2O-diluted sample.

Calibration Curve: Three aliquots of 0.5 ml of each urea nitrogen standard solution placed in sample cups are treated in the same way as for serum samples. A typical calibration curve thus drawn up is shown in Fig. 2.

Diluted Serum Method——Flow Diagram and Procedure: The flow diagram is the same as that of the direct serum method except a double heating coil is equipped in the heating bath to prolong the reaction time, and the manifold tubes in the proportioning pump are changed to suitable sizes as shown in Fig. 1b. 0.05 ml of serum is diluted with 0.45 ml of H_2O in a sample cup, and then treated in the same way as described in the direct serum method. The urea nitrogen value (mg/dl) is read on the calibration curve, prepared as described below.

Calibration Curve: Three aliquots of 0.5 ml of each urea nitrogen standard solutions placed in sample cups are treated in the same way as for diluted serum samples. The calibration curve thus obtained is shown in Fig. 3. The urea nitrogen value in serum calculated in mg/dl is obtained by multiplying the mg/dl of urea nitrogen standard solution by 10.

Result and Discussion

The reagent concentrations were first investigated to fit the reaction conditions of original manual method on the auto-analyzer.

10) All reagents used are JIS Reagent Grade except noted.
11) Purchased from Atlas Chemical Industries, Inc., New York.
12) Supplied from the Research Institute of Chugai Seiyaku Kabushiki Kaisha, Tokyo.
13) Technicon Instrument Corporation, Chauncy, New York.

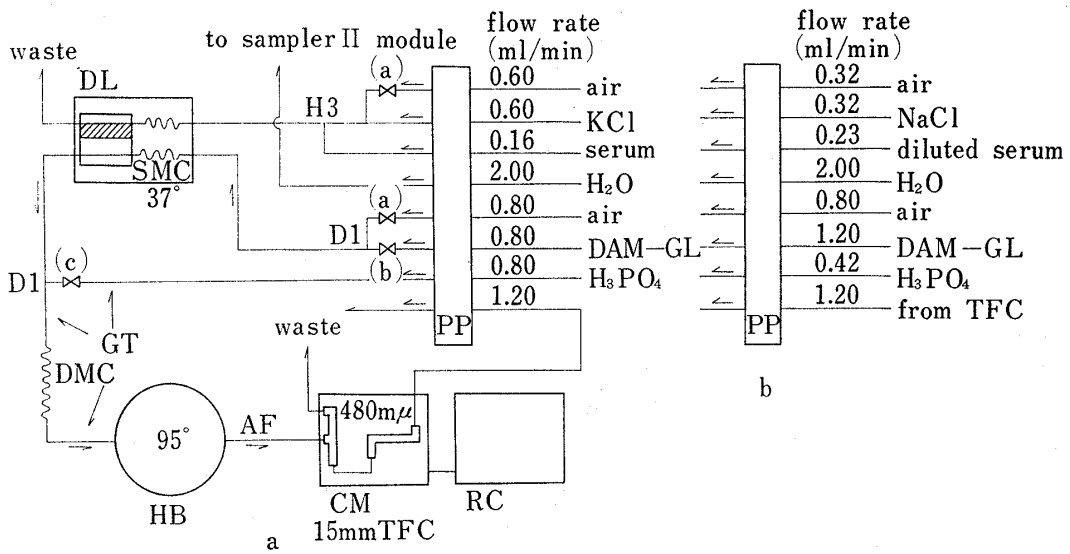

Fig. 1. Flow Diagram of the Present Methods

a: the direct serum method; b: manifold tubing in the diluted serum method
DAM-GL: diacetyl monoxime-glucuronolactone reagent; PP: proportioning pump; DL: dialyzer; SMC, DMC: single and double mixing coil, respectively; GT: glass tube; AF: acid flex tube; H3, D1: cuctus numbers; CM: colorimeter; RC: recorder; TFC: tubular flow cell; HB: heating bath; -▷◁-: pulse suppressor, (a) PS-4 orange-black, (b) PS-2 orange-green, (c) PS-3 orange-yellow

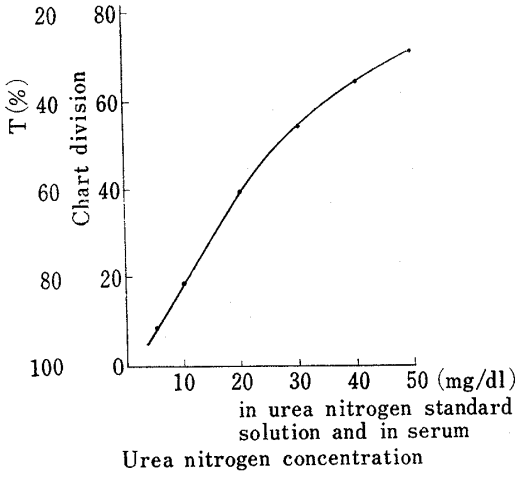

Fig. 2. Calibration Curve for the Direct Serum Method

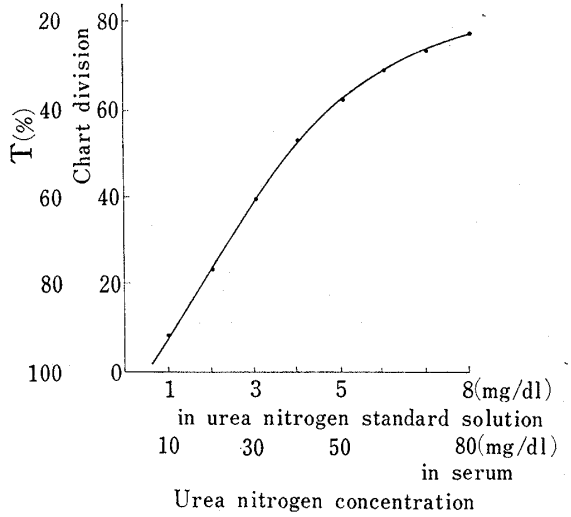

Fig. 3. Calibration Curve for the Diluted Serum Method

The concentration of diacetyl monoxime weakly affects the color development in the range of 0.5—3.0% in the direct serum method and 0.2—1.5% in the diluted serum method, and the described concentrations, 2% in the direct serum method and 0.5% in the diluted serum method give maximum intensities, respectively. D-Glucuronolactone plays as a stabilizer of the developed color in the reaction mixtures, and gives a moderate color intensity even in a lower concentration of urea. But the increasing concentration of the lactone slightly depresses the color development and increases the blank value. Consequently, the concentrations of 1% in the direct serum method and 0.5% in the diluted serum method were selected as the optima for color stability and low blanks.

It was recommended to add a large amount of an inorganic salt in order to reduce the water vapor pressure of the reaction mixture in the coil of heating bath, preventing confusion of babble pattern.[4] Potassium chloride is used for the purpose by adding in the diacetyl mono-

xime-glucuronolactone reagent in the direct serum method. The color intensity increases with increasing concentration of the salt.[14] This phenomenon might be occured by promoting dialysis of urea against the reagent solution in the dialyzer. However, a potassium chloride concentration higher than 5% in the reagent solution causes the calibration curve to become flat in a higher concentration of urea nitrogen, and hence 5% was selected as the maximum. In the diluted serum method, sodium chloride added in the diacetyl monoxime-glucuronolactone reagent was found to be more advantageous over potassium chloride for yielding an intense color. A higher concentration of the salt produces a more intense color, but causes turbidity in the reagent solution in the presence of Brij–35. The selected concentration, 15%, gives the maximum color intensity without turbidity.

The phosphoric acid concentration has a relatively large effect on the color development. A 50% solution of the acid gives the most intense color in the direct serum method, but makes the calibration curve sigmoidal. Finally, a 35% solution is selected to give an appropriate calibration curve for reading normal–abnormal levels of urea nitrogen in serum, which is assumed to be a straight line up to about 20 mg/dl urea nitrogen. In the diluted serum method, on the other hand, a higher concentration of phosphoric acid yields a more intense color, but a solution higher than 77% confuses the babble pattern in the flow system. Consequently, a 68% solution was selected to show a straight calibration curve up to 35 mg/dl urea nitrogen in serum.

Potassium chloride and sodium chloride solutions were employed as sample carriers in the sampling system in the direct and diluted serum methods, respectively. Both salts do not affect the color development over the concentration range of 0.5—1.5%, and each 0.9% was selected as usual automated methods.

Incorrect value of urea nitrogen caused by contamination of foregoing sample (carry over) has sometimes been observed at a high rate of sampling and/or in the use of a heavy reagent such as sulfuric acid or phosphoric acid. This phenomenon was also observed in the present methods when samples were taken into the system at the rate of 60 samples/hr. At the rate of 40 samples/hr, however, repeating determinations of urea nitrogen of 10.0 mg/dl following one of 50.0 mg/dl gave an increased value less than 0.5 mg/dl in each determination, suggesting that the carry over was negligible at this sampling rate in the present methods.

It has been considered that uneven amount of sample placed in each sample cup affected the value obtained in an automated method. The effect was not observed in the serum volume range of 0.4—1.5 ml, and so about 0.5 ml of serum is enough to give an exact value in the direct serum method. In the diluted serum method, serum is so exactly diluted with water in a sample cup as described in Experimental, that unevenness of sample do not arise.

Recovery tests were performed by adding known amounts of urea to serum samples. Satisfactory results were obtained as shown in Table I.

The results of parallel tests with the original manual methods[9] on serum samples are shown in Fig. 4 and 5. There were excellent agreement between the two methods in both cases of the direct and diluted serum methods.

Substances other than urea which might be occured in serum do not influence the color development, even in a concentration of 10 mg/dl. Those tested were creatine, creatinine, uric acid, ammonia, histamine, choline, chondroitine, glutathione, nicotinic amide, thiamine, lactic acid, 3-hydroxybutyric acid, pyruvic acid, oxaloacetic acid, 2-oxoglutaric acid, ascorbic acid, inositol, and 17 different α-amino acids. Citrulline gives a weak color with the methods in a concentration of 10 mg/dl. Triptphane reduces the value of urea nitrogen if present in serum in a concentration of 10 mg/dl. They give practically no interference on the methods in a normal range of their concentrations in serum.

14) In the original manual method, an increase of color intensity was not observed when the reaction was carried out in the presence of large amounts of potassium chloride.

Table I. Recovery of Added Urea

Serum No.	Initial urea nitrogen (mg/dl)	Urea nitrogen added (mg/dl)	Total urea nitrogen (mg/dl)	Recovery (%)
Direct serum method				
1	14.4	14.8	28.5	95.3
2	14.1	21.6	35.3	98.2
3	14.7	18.8	32.8	96.3
4	16.5	14.2	31.3	104.2
5	14.9	13.9	28.9	100.7
6	13.7	16.7	30.0	97.6
7	15.2	13.0	28.0	98.5
8	12.4	14.4	26.8	100.0
9	15.8	19.6	35.1	99.0
10	22.9	18.9	40.8	94.7
			mean	98.5
Diluted serum method				
11	16.3	36.0	52.5	101.2
12	11.7	36.0	47.4	97.4
13	13.6	36.0	49.8	101.5
14	9.6	36.0	45.4	97.9
15	12.9	36.0	48.6	97.8
16	13.2	36.0	49.6	103.0
17	16.4	36.0	52.6	101.2
18	12.6	36.0	48.6	100.0
19	17.4	36.0	52.7	96.0
20	14.1	36.0	50.2	100.7
			mean	99.7

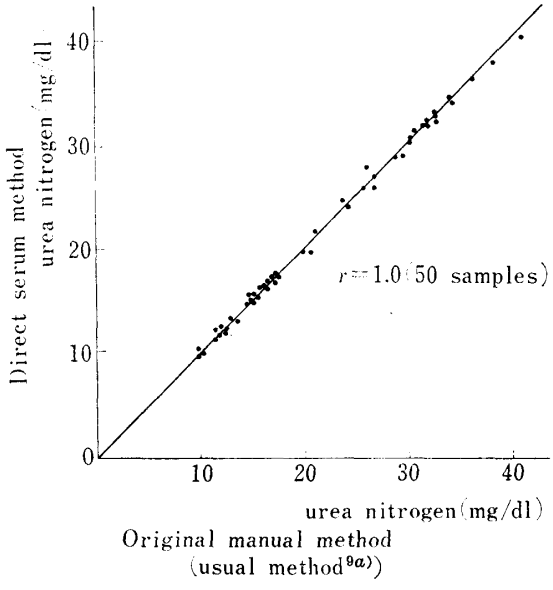

Fig. 4. Correlation between the Values obtained by the Direct Serum Method and the Original Manual Method

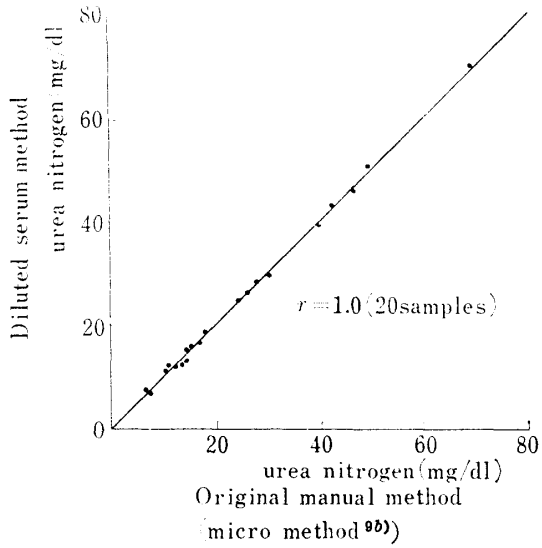

Fig. 5. Correlation between the Values obtained by the Diluted Serum Method and the Original Manual Method

The precision of the methods was studied with respect to repeatability and reproducibility. The repeatability was examined by performing 40 analyses on serum at the same time, separately with the direct and diluted serum methods. The standard deviations were

0.5 mg/dl (coefficient of variation: 3.5%) for mean value of 14.3 mg/dl of urea nitrogen in the direct serum method, and 0.3 mg/dl (coefficient of variation: 1.1%) for the value of 27.2 mg/dl in the diluted serum method. The reproducibility was obtained by repeating the procedure 40 times on each present method on serum of elevated urea nitrogen level (20.7—27.6 mg/dl). The values obtained for each serum agreed within ±2% in the direct serum method and ±1% in the diluted serum method.

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