The Automated Thiosemicarbazide-Diacetyl Monoxime Method for Plasma Urea

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Sensitivity and linearity of the diacetyl monoxime reaction for urea is greatly improved by the addition of thiosemicarbazide. Evidence is presented for the high specificity of this composite reagent.

IN RECENT YEARS the automated diacetyl monoxime (DAM) reaction as adapted to the AutoAnalyzer has become the method of choice in many clinical laboratories for the determination of urea in biologic fluids. The method is based on the condensation of urea with diacetyl, the latter usually being liberated from the monoxime in the presence of one of various acid reagents (*1, 2*). The reaction, however, does not follow Beer's law, and the colored product has been reported to be photosensitive (*3, 4*). By the addition of thiosemicarbazide (TSC) to the diacetyl reagent, Coulombe and Favreau (*5*) obtained linearity of response with a stable pink color whose E_{max} was shifted to 535 nm; the mixed reagent was reportedly stable at room temperature. Pellerin (*6*) automated the method, reporting exact linearity up to concentrations of 50 mg/100 ml. Marsh *et al* (*7*) compared manual versus automated methods employing TSC. In this comparison they modified both acid and DAM reagents somewhat, noting that color intensity of the product formed was proportional to concentrations of acid, DAM, and (to an optimum for any given mixture) TSC; linearity was not mentioned. We have confirmed the general observations of the latter authors but found that the recommended composition of acid reagent (0.05% w/v $FeCl_3$ in a mixture of 8% by volume of concentrated sulfuric and 1% by volume phosphoric acid) yielded very low color intensities, which could be increased many times over by using more conventional concentrations of ferric alum acid reagent.

For automatic logging and computation in a multichannel system, our requirements were precise linearity over the concentration range

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0–50 mg/100 ml, sensitivity to span 80% of the recorder scale, high reproducibility, and freedom from noise. The DAM-TSC reaction can fulfill all of these requirements. Sensitivity may be adjusted by the concentration of DAM employed. Results with the TSC automated method as used in this laboratory for the past 2 years were compared with those of the AutoAnalyzer N-1b method, which recently has been modified (N-1c) by use of TSC-DAM. Annino (*8*) also has reported results with a method that uses a ferric chloride acid reagent similar to that of Marsh *et al,* but with greatly increased concentrations of DAM and TSC in the color reagent.

Reagents

Ferric alum acid reagent Two volumes of the stock acid solution described in Technicon N-1b methodology are diluted with one volume of distilled water.

Stock diacetyl monoxime (DAM), 2.5% (w/v) A total of 25 g of reagent grade 2,3-butanedione-2-oxime (Eastman No. 86) are dissolved in a liter of distilled water. The diacetyl monoxime solution should be colorless. The presence of a gold color in the stock solution tends to increase noise in the automated system. Life is about 2 weeks.

Stock thiosemicarbazide (TSC) solution, 0.25% (w/v) A total of 2.5 g of thiosemicarbazide (Eastman No. 1275) is dissolved in distilled water. Life is about 2 weeks.

Working DAM-TSC solution A total of 60 ml of stock diacetyl monoxime solution is combined with 30 ml of stock thiosemicarbazide solution and diluted to 800 ml with 0.9% sodium chloride solution. The working reagent should be prepared daily in order to prevent precipitation of the reagent and the development of a gold color in the solution. For assays covering the range up to 150 mg/100 ml, the volume of stock DAM is reduced to 30 ml in preparing the reagent.

Urea standards Prepared by appropriate dilution in 0.01 N H_2SO_4 from stock urea solution containing 10.0 mg/100 ml of urea nitrogen. The use of phenylmercuric acetate as a preservative, suggested by Technicon, has not been investigated.

Procedure

The flow diagram for the automated TSC-DAM method is the same as the N-1b and N-1c methods of Technicon. The samples may be run at the rate of 40 or 60/hr with excellent washout between samples at a

standard 2:1 cam ratio. The sample (serum or standard) is diluted with 0.9% saline and is dialyzed at 37° against the mixed DAM and TSC reagent. The mixture of sample and TSC-DAM is brought back to the manifold where the diluted ferric alum-acid reagent is added and mixed thoroughly in a double mixing coil. The color is developed in a 1.6-mm (ID) 40-ft glass coil at 95° and the absorbance measured at a wavelength of 520 nm; the old 480-nm filter gives equivalent results with a slight decrease in sensitivity.

Conditions of Assay

High salt concentration in the DAM pickup solution does not appear to serve any useful purpose and may, in fact, contribute to osmotic shifts of water across the dialyzing membrane; in the present method, the concentration of NaCl has been reduced to 0.9%.

TSC added to DAM reagent linearizes the response with concentration so that the addition of urea to the pumped reagent becomes unnecessary. With increasing concentrations of TSC, the response curve straightens out first in the lower range and then linearizes at the higher end. For the final reagent described, linearity extends through concentrations of 100 mg/100 ml, although absorbance values exceed 1.0 (less than 10% T).

Sensitivity of response is governed directly by concentrations of both DAM and acid reagent. The final concentration of DAM employed here provides a usable concentration range of 0–60 mg/100 ml. This range may be doubled by halving the concentration of DAM in the reagent. The N-1b Technicon ferric alum acid mixture is used widely and works satisfactorily in releasing active diacetyl reagent, but the resultant sensitivity is quite high, and simple aqueous dilution of the reagent can adjust this satisfactorily.

Comparison of Results

Fifty-six plasma specimens, ranging in concentration of urea nitrogen from 9.0 to 56.4 mg/100 ml, were analyzed by both methods, the N-1b assay yielding results averaging 0.57 mg/100 ml (range 0–1.5) higher than with TSC-DAM. This discrepancy might have been ignored except for the fact that with certain extensively referenced pooled serum specimens we consistently obtained values with TSC-DAM 0.5–1.0 mg/100 ml lower than the N-1b results of other laboratories. To investigate the possibility that the TSC-DAM reagent might be yielding falsely low results, a series of serum and plasma specimens ranging in value from 15 to 56 mg/100 ml were subjected to two con-

secutive incubations with urease and rerun by both methods at increased sensitivity. With the Technicon method, discrete blank peaks were observed with the completely digested specimens which were absent in the TSC-DAM record. By extrapolation, the heights of these non-urea peaks were estimated as lying between 0.5 and 1.0 mg/100 ml. Since the response of the TSC-DAM method decreases linearly to zero concentration at the reagent base line, it is concluded that the normally small nonurea interference in serum or plasma has in some way been abolished by the use of TSC.

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