Direct Fluorometric Determination of Urea in Urine

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In this quantitative fluorometric method, diacetylmonoxime is used for the determination of the urea. The products of the reaction of urea with diacetylmonoxime in acid solution exhibit two fluorescence maxima, at 410 and 525 nm. The intensity of the 525 nm maximum is linear over a wide range of urea concentration and the reaction is shown to be practically specific for urinary urea.

Additional Keyphrases urea--diacetylmonoxime reaction • fluorometry vs. colorimetry

Urea in biological fluids has been determined by a variety of direct and indirect procedures. Some indirect methods depend on the hydrolysis of urea with the enzyme urease to form ammonia, with subsequent quantitation of the ammonia by nesslerization (1-4). Undesirable features of these indirect methods are the use of relatively unstable reagents and loss of ammonia during the procedure.

Direct determination of urea depends on the condensation of urea with some specific reagent such as α-isonitrosopropiophenone (5) and diacetyl derivatives (6-15) in acid media. The direct reaction between urea and diacetyl or diacetyl derivatives appears to offer distinct advantages for urea determination.

The products of the reaction of urea with diacetylmonoxime are fluorescent (16). The fluorescence maximum at 415 nm on optimal excitation at 380 nm has been used for the quantitative determination of urea. However, the fluorescence intensity of the reaction products at this wavelength is not directly proportional to the concentration of urea.

I have further examined the reaction of urea with diacetylmonoxime with respect to fluorescence maxima of the products, sample concentration, specificity, and kinetics. The improved method was then applied to determine urea in human and primate urine.

Materials and Methods

Apparatus

Fluorescence was measured on an Aminco-Bowman Spectrophotofluorometer, with use of a xenon lamp and an RCA 1P 28 photomultiplier detector.

Reagents

Phenol color reagent. 5.0 g of phenol and 25 mg of sodium nitroprusside were dissolved in water and diluted to 500 ml with ammonia-free distilled water. The reagent is not stable even at 4°C and must be prepared freshly before use.

Alkaline hypochlorite reagent. Sodium hydroxide, 2.5 g, and 0.21 g of sodium hypochlorite were dissolved in water and diluted to 500 ml with ammonia-free water.

Diacetylmonoxime solution. Five grams of reagent-grade diacetylmonoxime was dissolved in 500 ml of distilled water, 150 g of sodium chloride was added, and the solution diluted to 1 liter with water.

Urea nitrogen stock standard (urea nitrogen, 1 mg per ml). 2.14 g of reagent grade urea was dissolved in 100 ml of 5 millimolar sulfuric acid.

Urea solution, 10 mmol/liter. Urea, 0.6 g, was dissolved in 1.0 liter of ammonia-free water.

Ammonia stock standard, 40 mmol/liter. This was prepared by dissolving 2.641 g of ammonium sulfate in 1 liter of ammonia-free water; 10 and 20 mmol/liter solutions were prepared by appropriate dilutions of the 40 mmol/liter ammonia stock standard.

Commercial urine sample. Freeze-dried human urine samples were obtained from the Hyland Laboratories, Costa Mesa, Calif. 92626, and reconstituted with water according to the directions given.

Primate (Rhesus) urine samples. Pooled 24-h specimens of primate urine were provided by Dr. Nello Pace of the University of California, Berkeley.

Urease. A 100-mg pellet of urease (ICN Nutritional Biochemicals, Cleveland, Ohio 44128) was dissolved in 50 ml of water containing 500 mg disodium ethylenediaminetetraacetate. The enzyme solution was prepared just before use.

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Procedure

For the colorimetric determination of urinary ammonia and urea the method was adapted from that of Fawcett and Scott (18).

Procedure for determination of ammonia. Phenol color reagent, 5 ml, was added to each test tube containing 10 µl of the sample and a blank and the contents were mixed. Ammonia-free distilled water was used as a blank. Alkaline hypochlorite solution, 5 ml, was added immediately to each tube and the contents were thoroughly mixed. The tubes were placed in a beaker of water at 60°C for 15 min and the absorbance then measured at 630 nm with the Cary Model 14 spectrophotometer.

Procedure for urea. Ten microliters of the appropriately diluted urine samples was mixed with 50 µl of buffered urease and incubated at 37°C, with shaking, for 15 min along with an appropriate urea standard solution. After cooling to room temperature, the samples were treated as for the ammonia determination.

Fluorometric procedure. Urea sample, 0.1 ml, is pipetted into a screw-cap test tube (16 × 125 mm) and diluted to 1.0 ml with sulfuric acid (5 mmol/liter). To each tube, 0.8 ml of diacetylmonoxime solution and 0.2 ml of concentrated sulfuric acid are added. The tubes are capped tightly, placed in a boiling water bath for 40 min, then removed and allowed to cool to room temperature. A blank solution containing distilled water and urea standard solutions are processed in the same manner and used to prepare the standard curve. The fluorescence intensities are measured in the spectrophotofluorometer either at the emission maximum at 525 nm with an excitation wavelength of 380 nm or at the excitation maximum at 380 nm with an emission at 525 nm. The concentration of the unknown urea solution is determined from the standard curve.

Results and Discussion

Reaction of urea with diacetylmonoxime. The reaction of diacetylmonoxime with urea is not well understood. Natelson et al. (10) found the active reagent to be diacetyl, not diacetylmonoxime, whereas Hoseney and Finney (17) found that diacetylmonoxime produced greater color intensity than diacetyl. The reaction products of diacetylmonoxime and urea have an absorption maximum at 450 nm, as measured with a Cary Model 14 spectrophotometer. This absorption band has been used by various authors in determining urea. The compounds produced by the reaction of urea with diacetylmonoxime fluoresce at a wavelength of 410 nm when excited at 380 nm, as observed by McCleskey (16). However, the same reaction mixture also fluoresces at another wavelength maximum of 525 nm when the compound is activated at 380 nm. Figure 1 illustrates fluorescence intensity at the two different wavelength maxima vs. various concentrations of urea.

The fluorescence maximum of the reaction products with urea at 410 nm can be observed when either diacetyl or diacetylmonoxime is used as the substrate, but the fluorescence maximum at 525 nm is prominent only when a large excess of either reagent is used. The fluorescence at 410 nm is proportional to the amount of urea only at relatively low urea concentrations and it is strongly influenced by scattering background at 380 nm. The fluorescence intensity at 525 nm, on the other hand, is proportional to a much wider range of urea concentration. This probably is the result of decreased scattering interference, because the 525 nm peak position is relatively remote from the exciting wavelength.

The limit of detectability of urea by the fluorometric method is a few nanomoles and it is at least an order of magnitude more sensitive than the colorimetric method with the same reagents.

Effects of temperature. The reaction products that fluoresce at 525 nm vary with both time and temperature. As shown in Figure 2, their rate of formation at 100°C is markedly higher than that at 60° and 80°C. When the mixture is heated at 100°C, the reaction is essentially complete within 40 min.

Specificity. The reaction with diacetylmonoxime apparently leads to fluorescent products for compounds containing the system RHCO(S)NR′R* where R is hydrogen or a simple aliphatic radical, R′ is hydrogen, a simple aliphatic radical, or a phenyl group, and R* is less complex than a phenyl group (6, 17). The speci-
ficity of this reaction for urea has been studied by treatment of samples with and without the enzyme urease. When urea or human urine were incubated with an appropriate amount of urease at 37°C for 15 min, the mixtures were completely devoid of fluorescence, whereas untreated samples exhibited the proportional increase of fluorescence with increased concentration of urea or of urine, as shown in Figure 3.

Quantitative recovery of urinary urea. The concentration of urea in the Hyland Laboratory standard urines and in primate urine, as determined by this procedure, is shown in Table 1. I found mean values of 212.8 μmol of urea per ml for the Hyland urine and 121.9 μmol/ml for the primate urines. This value for urea in the Hyland urine agrees very closely with their value of 6 mg of urea nitrogen per milliliter. Analytical data given by Hyland Laboratories were obtained by absorbance measurements after the nesslerization of urease-treated samples. Excellent agreement between the two methods was also obtained for 120 μmol of urea per milliliter of the primate urine.

Precision. I determined the reproducibility of the analysis by use of two different concentrations of urea. The results of 10 replicates for 75.0 and 100.0 μmol of urea per liter indicate a value of 3.7 to 4.7% deviation within a confidence level of 95%. The precision of the fluorometric method in the analysis of urea is thus reasonably high and reproducible.

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References


