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Application of a Novel 1,1′-Dimethylferricinimum Dye for the Determination of Uric Acid in Urine

KEITH B. MALE, JOHN H. T. LUONG,* AND MICHAEL TRANI

Biotechnology Research Institute, National Research Council Canada, Montreal, Quebec, Canada H4P 2R2

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ABSTRACT

Water-soluble 2-hydroxypropyl-β-cyclodextrin (Hp-β-CyD), a cyclic and nonreducing oligosaccharide was used to enclose a hydrophobic guest molecule 1,1′-dimethylferrocene (DMF) to form a water-soluble yellow complex. At high concentrations (300 m*M*), Hp-β-CyD enclosed up to 100 m*M* DMF. The yellow complex was electrochemically oxidized (platinum vs Ag/AgCl poised at +450 mV) to form a blue dye, 1,1′-dimethylferricinium (DMF^+). This is a one-electron transfer process and the ferricinium cation formed exhibited an absorption peak at 650 nm. The concentrated DMF^+ was stable for at least 4 mo at 4°C and insensitive to a wide pH variation (pH 2–11).

Application of the novel DMF^+ complex as a colorimetric dye for the determination of uric acid in urine was successfully demonstrated. The reaction between the dye and uric acid is almost instantaneous and decrease in absorbance caused by the reduction of 1,1′-dimethylferricinium to 1,1′-dimethylferrocene can be followed at 650 nm. The results obtained agreed well with those of the reference reversed-phase HPLC method.

Index Entries: Uric acid; 1,1′-dimethylferricinium (DMF^+); 1, 1′-dimethylferrocene (DMF); 2-hydroxypropyl-β-cyclodextrin (Hp-β-CyD); inclusion complex; colorimetric method; urine.

*Author to whom all correspondence and reprint requests should be addressed.

INTRODUCTION

In humans, uric acid is the principal product of the catabolism of adenosine, guanosine, and other purine nucleotides. Determination of uric acid in blood or urine is of clinical importance since several diseases such as gouty arthritis and uric acid urolithiasis are known to be associated with abnormal levels of uric acid. Hyperuricemia is defined by serum or plasma uric acid concentrations higher than 7 mg/dL and 6 mg/dL for males and females, respectively (*1*). Detection and follow-up of asymptomatic hyperuricemia are of importance because many patients are at risk for renal disease, which may develop as a result of hyperuricemia and hyperuricosuria. Although less common, hypouricemia is defined as serum urate concentrations below 2 mg/dL (*1*). Severe hepatocellular diseases with reduced purine synthesis or xanthine oxidase activity and Fanconi's syndrome, a defective renal tubular reabsorption of uric acid are two typical examples of hypouricemia.

Three different methods have been used for the determination of uric acid. The phosphotungstic acid (PTA) method develops a blue color (tungsten blue) upon reduction by urate in alkaline medium (*1*). The intensity of the color change is then followed spectrophotometrically at 650–700 nm. The PTA method is subject to many endogenous as well as exogenous interferences and the presence of protein causes turbidity and quenching of the absorbance. To date, attempts to modify the PTA method have had little success in improving its specificity (*1*). The second method relies on high-performance liquid chromatography equipped with reversed-phase columns. Although this procedure is sufficiently specific to serve as a reference method (2), it is expensive and requires skillful personnel as well as capital investment.

An enzymatic assay referred to as the uricase method employs uricase to catalyze uric acid to allantoin and hydrogen peroxide. The decrease of absorbance as urate is oxidized can be followed spectrophotometrically at 282–292 nm (*3*). The coupled uricase method utilizes the hydrogen peroxide produced as substrate for a biochemical reaction involving peroxidase or catalase (*4*). For instance, hydrogen peroxide is allowed to react with ethanol in the presence of catalase to produce acetaldehyde, which then reacts with NAD^+ in the presence of aldehyde dehydrogenase to form acetate and NADH. Increase in absorbance owing to the formation of NADH can be monitored at 340 nm. Another example of the coupled uricase method is the formation of formaldehyde from the reaction of hydrogen peroxide with methanol in the presence of catalase. The formaldehyde produced is then reacted with acetylacetone in the presence of ammonia to produce 3,5-diacetyl-1,4-dihydrolutidine, a chromophore with a strong absorption at 410 nm. The major drawback of these techniques is the cost of enzymes and other cofactors involved although it is inherently more specific than the PTA method.

In this study, the ability of 2-hydroxypropyl-β-cyclodextrin (Hp-β-CyD) to enclose 1,1′-dimethylferrocene (DMF) was exploited to develop a spectrophotometric assay for uric acid in urine. In general, cyclodextrins, known as cyclic and nonreducing oligosaccharides consisting of glucose units linked by α-1,4 bonds, can form inclusion complexes with various organic chemicals by incorporating such guest molecules into their hydrophobic cavities (*5,6*). Hp-β-CyD appears to be a potential choice since it possesses a very high solubility (50% w/v in aqueous media) and a cavity size of 7.8 Å in diameter and 7.8 Å in depth. Water-insoluble ferrocene [*bis* (η^5-cyclopentandienyl) iron] and its derivatives including 1,1′-dimethylferrocene have been studied extensively to develop mediated biosensors because of their electron-exchange properties with various enzymes (*7,8*). Ferrocene has been shown to possess a pentagonal antiprismatic structure in the crystal state. The mean iron–carbonyl distance is approx 2.04 Å, whereas the carbon–carbon distance is near 1.40 Å and these dimensions are expected to be similar for 1,1′-dimethylferrocene (*9*). In view of this, Hp-β-CyD should be able to enclose DMF to form a water-soluble complex. The electrochemical oxidation of DMF to the corresponding cation and some characteristics of this dye were also presented and discussed.

MATERIALS AND METHODS

Chemicals

All chemicals used were of the highest purity available. 2-Hydroxypropyl-β-cyclodextrin (Hp-β-CyD), ammonium formate, and dodecyltrimethylammonium bromide were purchased from Aldrich (Milwaukee, WI). 1,1′-Dimethylferrocene (DMF) was obtained from Polysciences (Warrington, PA). Uric acid, ascorbic acid, glutathione, acetaminophen, caffeine, β-D-glucose, gentisic acid, theobromine, and theophylline were purchased from Sigma (St. Louis, MO). Cysteine was obtained from BDH (Poole, England), whereas aspirin (source of acetylsalicylic acid, ASA) was purchased from a local drug store.

Dissolution of 1,1′-Dimethylferrocene in Hp-β-CyD

Solutions of Hp-β-CyD were prepared in 20 m*M* acetate, pH 5.2 buffer to a maximum concentration of 300 m*M*. 1-1′-Dimethylferrocene was solubilized in various concentrations of Hp-β-CyD by constant stirring for 2 h. The concentration at maximum solubility was determined spectrophotometrically (Beckman DU-640) using the absorption coefficient of 100 $cm^{-1}M^{-1}$ for 1,1′-dimethylferrocene at 435 nm.

Preparation of 1,1'-Dimethylferricinium

Solutions (25 mL) of 1,1'-dimethylferrocene (approx 90 m*M*) solubilized in Hp-β-CyD (250 m*M* in 100 m*M* KCl, 100 m*M* acetate, pH 5.2) were electrochemically oxidized at a platinum foil electrode poised at +450 mV vs an Ag/AgCl reference electrode in a three-electrode system. The counter and working electrodes were separated by a 2*M* KCl bridge and the electrochemical oxidation was performed using a polarographic analyzer/stripping voltammeter (Princeton Applied Research, Princeton, NJ). During the course of oxidation, the current was recorded and the absorbency of 1,1'-dimethylferricinium formed was monitored at 650 nm. The absorption coefficient for this cation was determined by adding subsaturating known concentrations of reducing agents such as ascorbic acid or uric acid. From this value the final concentration of 1,1'-dimethylferricinium could be calculated as well as the percentage conversion for the electrochemical oxidation from the starting solution.

The HPLC System and Uric Acid HPLC Standards

The high-performance liquid chromatography system (HPLC, model Maxima 820, Waters Scientific, Bedford, MA) is equipped with a solvent delivery system (model M 590), a sample processor (Model WISP 710 B) and a spectrophotometer (model M 481). The HPLC column used was Supelcosil LC-18 (150 × 4.6 mm) packed with 3 μm octadecylsilane material (Supelco, Oakville, Ont., Canada). The mobile phase was 1 m*M* ammonium formate, 7 m*M* dodecyltrimethylammonium bromide and 40% methanol (HPLC grade). The flowrate was 0.9 mL/min and the column temperature was maintained at 35°C. All chromatograms were monitored at 265 nm and the solvent front appeared after approx 2.3 min. In addition to uric acid with a retention time of 8.6 min, the reversed-phase HPLC system also detected cysteine, acetaminophen, and ascorbic acid with a corresponding retention time of 2, 2.5, and 9.6 min, respectively.

From a 1 m*M* stock solution of uric acid in 10 m*M* sodium hydroxide, dilutions were made with 10 m*M* NaOH to yield concentrations of 12.5, 25, 50, and 75% of the initial uric acid stock solution. An injection of 10 μL of each uric acid standard was used for calibration of the HPLC column.

Measurement of Uric Acid in Urine

Urine samples were collected from ten healthy males (23–47 yr old) at three random sampling times (not fasting). Urine was diluted eightfold in 10 m*M* NaOH, and 10 μL of the resulting urine solution was delivered to the HPLC column.

For the determination of uric acid by the ferricinium dye method, the ferricinium dye was diluted fivefold in 100 m*M* borate buffer pH 9.3. The reference absorbance was monitored at 650 nm by further diluting the

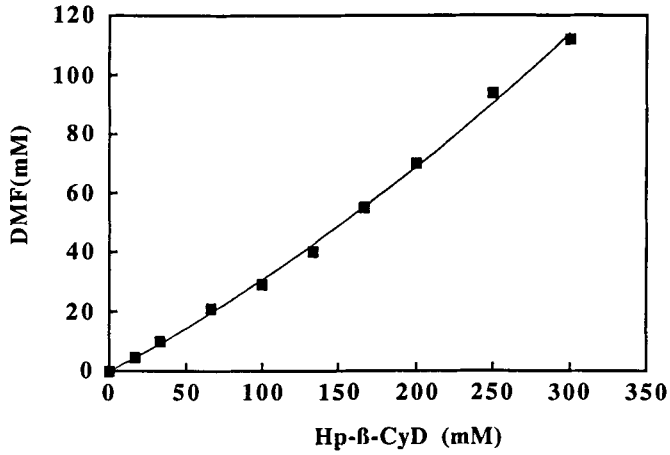

Fig. 1. Dependence of the dissolution of 1,1′-dimethylferrocene (DMF) on the concentration of 2-hydroxypropyl-β-cyclodextrin (Hp-β-CyD) in 20 m*M* acetate buffer, pH 5.2.

dye twofold with buffer. To a 500-μL sample of dye, 500 μL of urine was added and the absorbance at 650 nm was recorded immediately. The change in absorbance at pH 9.3 reflects the level of uric acid as well as other unknown reducing agents. To quantify the levels of such unknown reducing agents, a blank measurement was performed at pH 4.8 using 250 m*M* acetate buffer. Similarly, a reference absorbance was monitored by diluting the dye (fivefold diluted in the acetate buffer) twofold in buffer. After adding urine samples to the dye (1:1 ratio), the absorbance at 650 nm was monitored immediately. The difference of absorbance change at pH 4.8 represents the concentration of unknown reducing agents, which unlike uric acid will reduce 1,1′-dimethylferricinium at pH 4.8. The uric acid levels obtained were compared to those determined by HPLC.

RESULTS AND DISCUSSION

Dissolution of DMF in Hp-β-CyD

DMF was easily dissolved in methanol or chloroform and exhibited an absorption coefficient of 100 $cm^{-1}M^{-1}$ at 435 nm. Such a value was then used to estimate the solubility of DMF in Hp-β-CyD. DMF was only slightly soluble in aqueous solutions (<0.2 m*M*), but its solubility was greatly increased by the formation of inclusion complexes with the water-soluble Hp-β-CyD. If a known subsaturating concentration of DMF was solubilized in Hp-β-CyD (167 m*M*), the absorption coefficient obtained was still 100 $cm^{-1}M^{-1}$. Hp-β-CyD exhibits a high solubility (50% w/v) and as a result a 300 m*M* stock could be prepared. As shown in Fig. 1, the molar ratio of Hp-β-CyD to DMF in DMF saturated solutions was 3.7 at

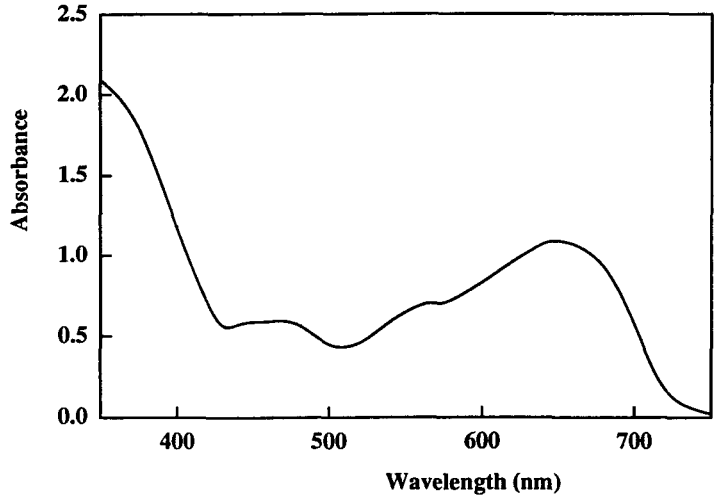

Fig. 2. Spectrum of 1,1′-dimethylferricinium in 100 m*M* acetate, 100 m*M* KCl buffer, pH 5.2.

the lowest concentration of Hp-β-CyD and the ratio decreased slowly to 2.7 at the highest concentration. Therefore, it was possible to prepare solutions of DMF as high as 100 m*M*.

Electrochemical Preparation of DMF^+

During the time course of electrochemical oxidation, the current dropped and the solution turned from yellow to green and finally to dark blue. The development of the blue color was monitored at the absorption maximum for DMF^+ at 650 nm (Fig. 2). It should be noted that the DMF-Hp-β-CyD complex did not absorb at this wavelength. After about 12–16 h, the absorbance reached a plateau while the current continued to decrease. For the preparation of DMF^+, if the reaction was allowed to proceed much further (>24 h), eventually an absorbance decrease was noted, indicating the breakdown of the oxidized product. The concentrated DMF^+ was stable for at least 4 mo when stored at 4°C. It was also noted that the DMF-Hp-β-CyD complex stored at room temperature began to turn green, indicating a preference toward the oxidized state. The ferricinium cation was insensitive to pH variation from 2–11. This characteristic is of advantage since the assay can be performed regardless of some fluctuation in pH.

Chemical Oxidation of DMF^+

DMF was also oxidized by ceric sulfate or iodine/KI to $DMFH^+$, known as 1,1′-dimethylferrocenonium (*10*). The reaction was almost instantaneous and the spectrum of this cation was identical to that of 1,1′-dimethylferricinium. However, the resulting products were not stable to

changes in pH and often precipitation occurred with time. Such results were not completely unexpected since in strong acid media ferrocene was reported to undergo extensive protonation on the metal atom (*9*). The NMR spectra of the cation in boron trifluoride hydrate exhibited high field resonance characteristics of metal bound protons. As also reported (*9*), in the protonated ferrocene the cyclopentadienyl rings are no longer parallel but are somewhat tilted so that the angle ω, defined by perpendiculars to each ring from the metal atom, lies between 135 and 180°. As a result of this finding, the chemical oxidation pathways were not pursued further.

Reduction of the DMF^+ Dye by Uric Acid and Interference Studies

Uric acid reduces DMF^+ instantly at pH above 8. By plotting uric acid concentration (m*M*) vs absorbance at 650 nm, a straight line resulted with a slope of 0.65 $OD_{650\ nm}$/m*M* with a correlation coefficient of 0.999 (n = 9, figure not shown). From this value, the absorption coefficient of the DMF^+ was estimated as 325 $cm^{-1}M^{-1}$ since two molecules of DMF^+ are required to oxidize each molecule of uric acid. At neutral pH, the rate of dye reduction by uric acid was very slow with an end point of only 0.30 for 1 m*M*, whereas below pH 6.0 uric acid completely lost its ability to reduce the dye. The absorption coefficient of 325 $cm^{-1}M^{-1}$ was also obtained if ascorbic acid was used as the reducing agent. However, ascorbic acid reduced the DMF^+ instantly at both pHs of 4.8 and 9.3. From the absorption coefficient, the stock solution of DMF^+ was determined to be 70–80 m*M*, corresponding to an 80–90% conversion from the starting DMF/Hp-β-CyD yellow complex.

In general, urine and plasma contain several endogenous and exogenous analytes other than uric acid which have been shown to interfere with the phosphotungstic acid (PTA) method (*1*). These interferents include endogenous substances such as glucose, ascorbic acid, glutathione, cysteine, and ergothionine as well as exogenous compounds such as acetaminophen, acetylsalicylic acid (ASA), caffeine, gentisic acid (a salicylate metabolite), theobromine, and theophylline.

All the analytes except for ergothionine which is not commercially available were tested with the new 1,1′-dimethylferricinium dye method at both pH 4.8 and 9.3. Glucose, caffeine, ASA, gentisic acid, theobromine, and theophylline have no reducing capabilities with respect to ferricinium at either pH 4.8 or 9.3.

Ascorbic acid reduces the dye at both pH 4.8 and 9.3 and, therefore, can be eliminated as an interferent by performing a blank at pH 4.8. Cysteine reduces the dye instantaneously at pH 9.3 for concentrations up to 3 m*M*, whereas the reduction was somewhat slower and concentration dependent at pH 4.8. Concentrations less than 0.5 m*M* reduce the dye in-

stantly and since the level of cysteine typically found in the urine (0.8–1.8 L urine/d) is only 0.08–0.80 mmol/d (*11*), cysteine can be eliminated as an interferent by performing a blank at pH 4.8. Like cysteine, glutathione also reduces ferricinium instantly at pH 9.3. However, glutathione reduction of DMF^+ was very slow at pH 4.8 so that the blanking method would not eliminate glutathione as an interferent unless the time for the blank assay was increased. Interestingly, both these analytes at 1 m*M* reduce the DMF^+ dye by 0.33 OD_{650nm} which would appear to indicate that the ratio of analyte to DMF^+ was only 1:1. Glutathione (2.2 m*M*) sometimes spills from the red blood cells into the plasma and could be a problem for the determination of uric acid in blood plasma (*11*). However, normal urine samples are not anticipated to have a significant amount of thiscompound. Acetaminophen reduces DMF^+ at pH 9.0 but not instantaneously. At the lower pH of 4.8 acetaminophen has no reducing capabilities, and therefore this analyte cannot be eliminated by the pH 4.8 blank and could pose a problem as an interferent if at significantly high levels.

Measurement of Uric Acid in Urine

Buffers of high ionic strength for the blank assay (250 m*M* acetate pH 4.8 and the test assay (100 m*M* borate pH 9.3) were used to ensure that the pH would be maintained after adding urine samples (the urine samples tested varied from pH 5–8). The concentration of uric acid in the samples tested varied from 1–8 m*M*. Samples containing uric acid concentrations greater than 5.0 m*M* were further diluted. Readings were recorded immediately as there was a tendency for the OD_{650} nm to drift downward and after a short time period the urine precipitated the complex. It should be noted that the normal physiological range for uric acid under fasting conditions is from 1.5–4.4 m*M*, depending on the rates of water intake and water loss (*11*).

The blank measurements varied from sample to sample, ranging from 0–30% of the reading obtained at pH 9.3. Undoubtedly, several endogenous as well as exogenous compounds that are capable of reducing the ferricinium dye were expected to be present in these urine samples. However, HPLC data confirmed that the tested urine samples did not contain any appreciable amount of common interferents such as acetaminophen, ascorbic acid, and cysteine. About 50% reduction in the blank readings was observed if urine samples were left at room temperature (approx 22°C) for 24 h. The level of uric acid remained the same indicating the stability of uric acid and the fact that the sample could be read at any time up to 24 h. The values obtained by the dye method were in excellent agreement with those of HPLC. The values of HPLC plotted against those of the dye method resulted in a straight line with a slope of 1.01 and a correlation coefficient of 0.96 (n = 29) (Fig. 3). Such good agreement thus validated the applicability of the DMF^+ method for measurement of uric acid levels in urine.

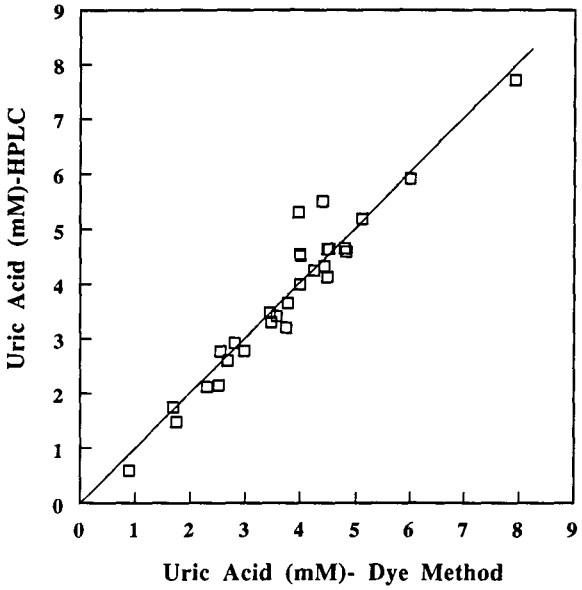

Fig. 3. Urinary uric acid level determined by HPLC and the 1,1′-dimethylferricinium dye method (C_{HPLC} = 1.01 C_{DMF+}, R^2 = 0.96).

C_{HPLC} = Uric acid concentration determined by HPLC
C_{DMF+} = Uric acid concentration determined by the dye method
R^2 = Correlation coefficient

In brief, a rapid and reliable colorimetric assay based on a 1,1′-dimethylferricinium dye has been successfully developed for the determination of uric acid levels in urine samples. The reaction between the dye and uric acid is almost instantaneous and decrease in absorbance owing to the reduction of 1,1′-dimethylferricinium to 1,1′-dimethylferrocene can be followed at 650 nm. Unlike the uricase or the coupled uricase enzymatic assay, this procedure requires no enzymes and other expensive cofactors. The method was also found to be more specific than the PTA method since many of the potential interferents would not reduce 1,1′-dimethylferricinium. The results obtained by the novel dye procedure agreed well with those of the reference reversed-phase HPLC method.

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