

Spectrophotometric Determination of Urea in Urine Samples by using Bispyrazolone Method

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Accurate quantization of urea is very essential for precise dialysis therapy in the case of kidney failure. In the present paper we report a new colorimetric method for urea quantification using urease as a probe. The method is based on the reaction between bispyrazolone and ammonia released by the urease catalyzed hydrolysis of urea. A linear relationship of the absorbance at 540 nm of the product was observed with urea concentration in the range of 1.5 µg-20 µg (slope = 0.0439; $R^2 = 0.998$). The present method was also analyzed for potential interferences and results obtained were compared with other popular methods of urea quantitation. Urine samples were analyzed by two existing methods namely diacetyl monoxime method and indophenol method, and their results were compared with that obtained by the newly developed bispyrazolone method. Results by this method and by other known methods were in good agreement. The present method has the potential for automation for high throughput screening of urine samples.

Key Words: Haemodialysis; Urea Quantitation; Spectrophotometric; Bispyrazolone; Urine Analysis

1. Introduction

An essential component of evaluating and improving global health is access to appropriate diagnostic tools [1]. Unfortunately current diagnostic tests are largely inadequate for meeting health demands in developing countries [1]. According to an extrapolation based on a survey in 2010, a total of 2 million people would be recognized as kidney patients requiring dialysis [2]. Determination of urea content in dialysis blood samples is unavoidable for diagnosing renal diseases [3, 4] and has significant impact on the general health and mortality of the kidney patients [5]. Determination of urea in biological fluids and in agro-foods is important. Urease-based biosensors have been used to determine urea content of blood serum, urine, natural water, wastewaters, foods, wines, etc [4, 6].

Currently, either the direct chemical methods or the indirect urease method are used for measurement of urea. The diacetyl monoxime method relies basically on the formation of an imidazolone, which gives rise to the formation of a red complex with thiosemicarbazide at high temperature [7, 8]. Additional deproteinization steps make this assay cumbersome. The diacetyl reagent is photo sensitive and does not follow the Lambert-Beer's law which makes it difficult to reproduce results. Addition of oxidants stabilizes the colour, while other reagents augment the color and thio semicarbazide minimizes the photosensitivity of the reaction [9].

Use of urease as a probe for estimation of urea in biological samples is a method of choice because of the selectivity of urease towards urea. The urease method involves enzymatic hydrolysis of urea,

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followed by assay of released ammonium by an additional procedure. Ammonia has been commonly quantitated by Nessler's reagent, fluorimetric analysis or Berthelot's reaction. Nessler's reagent tends to form precipitate on long standing when fluorimetric reagents are subjected to non-specific interferences. Use of coupled enzymes which uses ammonia as a substrate is often discouraged because of the various problems associated with these kinds of assays. Use of Berthelot's reaction has gained much importance because of its sensitivity and ease of execution but this method also has disadvantages. The objections to this method include requirement of high temperature, lengthy operational time, molecular heterogeneity of chromophore population, sensitivity to various non-specific interferences and buffer incompatibility. Use of light sensitive corrosive reagents raises environmental concerns and poses difficulty for automated analyzers [10]. In the absence of a potential spectrophotometric assay, urea analysis by colorimetric methods has become a compromise between the objective of the study and resources available at the site of diagnosis.

In the present work we report a new method for estimation of urea using urease as a probe. Urea present in urine samples was first hydrolyzed by urease, followed by chemical coupling of bispyrazolone reagent with the enzymatically generated ammonia in activated form. This reaction results in the formation of coloured rubazoic acid which is determined at 540 nm.

2. Materials and Methods

Chemicals

Jack bean urease (Type IX) with the specific activity of 30U/mg was procured from Sigma Chemical Co., St. Louis, USA. Urea (enzyme grade) used as standard was purchased from E. Merck, India. Diacetyl monoxime and p-dimethylamino benzaldehyde were obtained from SD-fine chemicals. Thiosemicarbazide, Phenol, Sodium nitroprusside, Sodium hypochlorite, Nessler's reagent and ferric chloride hexahydrate were procured from Qualigens, India. Bispyrazolone was obtained from Fluka as well as synthesized in the laboratory. All other reagents

were of analytical grade. All urine samples for urea analysis were collected from the volunteers with mixed age and sex groups.

Methods

3-Methyl-1-phenyl-5-pyrazolone was synthesized according to the procedure given in Ahluwalia and Aggarwal (2000) [11]. An equimolar mixture of ethyl acetoacetate (10 mL, 0.0783 mol) and phenyl hydrazine (7.5 mL, 0.076 mol) was condensed in a boiling water bath with constant stirring for 2 h. The reaction mixture was allowed to cool and 100 mL of diethyl ether was added and stirred vigorously. Solidification of reaction mixture started in a few minutes. It was washed with diethyl ether on a Buchner funnel to remove colored impurities. Product was recrystallized with ethanol:water (1:1, v/v) or hot water.

Bispyrazolone was synthesized according to the protocol of Kruse and Mellon [12]. Bispyrazolone was synthesized by placing the mixture of 17.4 g of 3-Methyl-1-phenyl-5-pyrazolone monomer (0.0172 mol), 25 g of phenyl hydrazine (0.0405 mol) and 100 mL of 95 % ethanol in a 250 mL round-bottomed flask and refluxing in water bath for 24 h. The insoluble bispyrazolone was filtered off at intervals of a few hours and washed with hot ethyl alcohol. Bispyrazolone was recrystallized from hot water.

All the melting points were recorded on Tropical Labequip apparatus and are uncorrected. UV spectra were recorded on Cecil as well as Shimadzu UV-Vis spectrophotometers equipped with printer facility. IR spectra were recorded on Perkin-Elmer FT-IR SPECTRUM-2000 and ¹H-NMR spectra were recorded on Bruker Spectrospin AVANCE (300 MHz) with TMS as the internal standard. The products were identified by IR, NMR and UV spectra.

Urease activity was assayed by using bispyrazolone method in triplicates. One enzyme unit was defined as the amount of enzyme required to liberate one μ mol of ammonia in one minute under the defined test conditions (37°C, 50 mM phosphate buffer, pH 7.5, containing 50 mM NaCl and 1 mM EDTA, 250 mM urea). Urea in the urine samples was

assayed in triplicates by both the direct and indirect approaches. Direct method includes the diacetyl monoxime method while the indirect quantitation was performed by using the Indophenol and Bispyrazolone methods. Urinary urea by Diacetyl monoxime method was quantitated by following the procedure of Wybenga *et al.* [13]. In indirect approach urine samples were analyzed in triplicate for urea content by allowing the urea hydrolysis by urease and the resultant enzymatically produced ammonia was measured by the indophenol method. Ammonia formed after ureolytic activity was determined according to the procedure given by Weatherburn [14] and by Witte and Medina-Escobar [15].

The urea content of urine samples was also measured by the newly developed bispyrazolone assay. In a 1 mL reaction mixture, suitably diluted urine samples in triplicates were incubated with a solution of urease (1 U-2 U) for 60 min at 37°C. After enzymatic reaction, further reaction was stopped by adding 250 µL of 0.5 M oxalic acid. Colour was developed by adding 500 µL of Chloramine-T solution (0.25 % prepared in 0.5 N NaOH) followed by addition of 2 mL of bispyrazolone reagent (0.5 mg/mL, 0.1 N NaOH). Colour develops immediately at room temperature (~37 °C) and the absorbance was recorded at 540 nm. A standard curve was prepared by incubating known urea concentration with urease in an initial 1 mL reaction mixture followed by the rest of the procedure.

3. Results and Discussion

Since urea is the most abundant organic solute in urine, it was one of the first substances to be determined in biological fluids. Measurement of urinary urea excretion has been suggested as a means of estimating nitrogen balance in hospitalized patients who are malnourished [16]. Urea is formed in our body by urea cycle enzymes from the amino acid nitrogen pool and released largely into urine (0.25-0.33 mols urea/day; 12-20 g/day).

The common methods for urea determination employ either the direct reaction of urea with diacetylmonoxime or involve indirect method estimating the ammonia released. In our study, the diacetyl

monoxime [13] was allowed to react with the known concentrations of urea and absorbance obtained at 540 nm was plotted as a function of urea concentration (Fig. 1). The workable range for urea determination was found to be 6 µg-90 µg (0.1-1.5µmols) of urea (Fig. 1). The slope and regression coefficient of the standard curve was found to be 0.0074 and 0.988 respectively. Although diacetyl monoxime method is quite popular among chemical methods and has been automated, it is rarely used because of interference from some amino acids (e.g. Citrulline) and even Creatinine. In urine particularly, some unidentified compounds were found to interfere with this method [17]. More importantly, the monoxime method has the disadvantage of utilizing toxic chemicals and requiring elevated reaction temperatures up to 100°C to develop the color complex.

On the other hand the urease based indirect methods are found to be more specific [18]. The urease method involves enzymatic hydrolysis of urea, with the released ammonium being assayed using indophenol [19-22], Nesslerization [23-25], pH indicator [10, 26], coupled enzyme assay [27, 28], ninhydrin [29] or by fluorimetric assay [30]. Although dry chemistry methods comprising pH indicators and fluorimetric method are promising for urea determination, chances of false positive results and

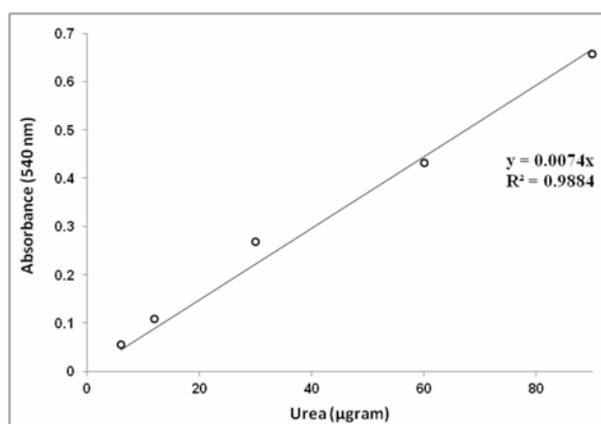


Fig. 1: Standard curve of urea quantitated by diacetyl monoxime thiosemicarbazide (DAM-TSC) method. Known amounts of urea were quantitated by DAM-TSC reagent in triplicates and the standard curve of urea was plotted by relating absorbance as a function of amount of urea

interferences make these assays incompatible for rigorous urea analyses [31]. For example, minute amounts of heavy metals produce a considerable inhibition in the latter method [32]. Nesslerization method also involves deproteinization and poses turbidity problems at higher levels of urea. Berthelot reaction is the only one still being used among the chemical reactions for ammonia estimation. Many commercial diagnostic kits available in the market are the translated form of the original Berthelot reaction. The indophenol method was standardized and results indicated that Lambert-Beer's law was valid for a limited range of 1 μg -6 μg (20-100 nmoles) of urea. The slope of the calibration curve was 0.1667 while the regression coefficient was found to be 0.9811 (Fig. 2). Although the color yield in this method is high, toxicity and corrosive nature of the reagents used during the assay create concern. Specific criticisms of the indophenol method include the presence of high and variable blanks and sensitivity. Moreover, indophenol is not the only product formed in Berthelot reaction, thus the treatment of different types of samples can provide different types of chromophores. Indophenol methods for urea quantitation also suffer from the drawbacks of buffer's incompatibility, non-specific coloration with urea and proteins.

The present method being described here involves the use of bispyrazolone as a reagent.

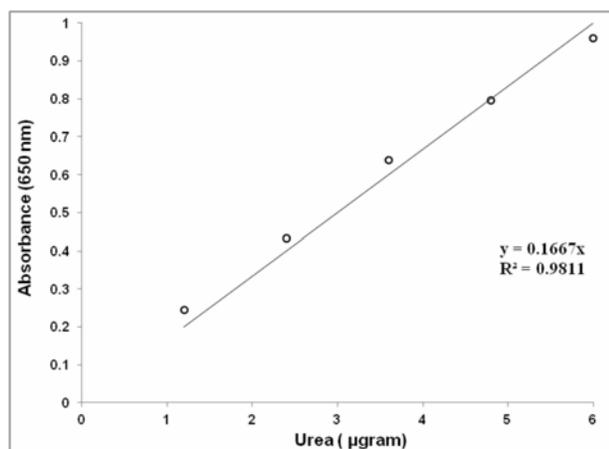
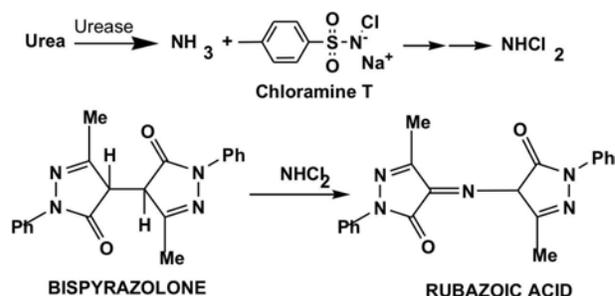


Fig. 2: Standard curve of urea determination by indophenol method. Known concentrations of urea in triplicates were incubated with urease, and ammonia obtained was quantitated by indophenol method

Ammonia produced as a result of ureolytic activity is used to convert it at alkaline pH to dichloramine which further reacts with bispyrazolone to yield rubazoic acid (Scheme 1).



Scheme 1: Reaction mechanism of the formation of rubazoic acid after urease activity

The reagent concentrations and order of addition were optimized (data not shown) and a standard curve is presented in Fig. 3. Beer's law (i.e. concentration versus absorbance readings are in direct linear proportion) was found to be obeyed over a wide range of urea concentrations (1.5 μg -20 μg ; 30-300 nmoles), thus reducing the possibility of error. The slope of the curve was 0.0439 while the coefficient of regression (R^2) value was 0.998. The upper limit of concentration of urea measurable by this bispyrazolone method was almost three times that by the indophenol method while the lower limits in

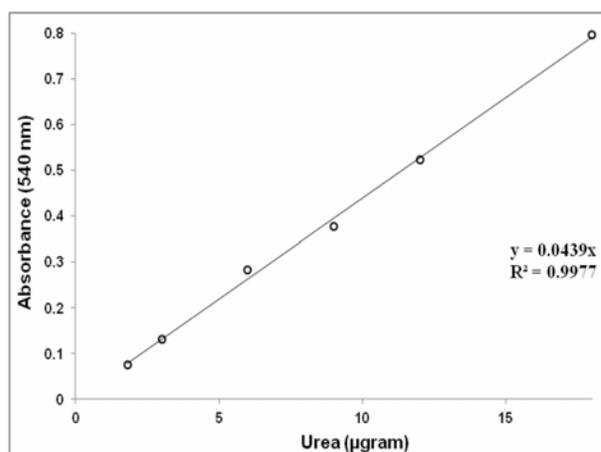


Fig. 3: Standard curve of urea determination by bispyrazolone method. Known concentrations of urea in triplicates were incubated with urease, and ammonia obtained was quantitated by bispyrazolone method

both the cases are comparable. The formation of purple color with high extinction coefficient value and insignificant blank reduces many of the problems encountered in the previously published assays. In the current method color develops instantly while in indophenol and diacetyl monoxime methods, the minimum time taken for colour development was 30 min. Significant improvement in the speed of execution is highly desirable for high throughput screening with large batches of samples. Both indophenol and bispyrazolone methods were checked for non-specific interference and the results are given in Fig. 4. As stated earlier indophenol method suffers from the development of many chromophores which further contribute to the non-specific color with urea. As evident from Figs. 4A and 4B, the present method

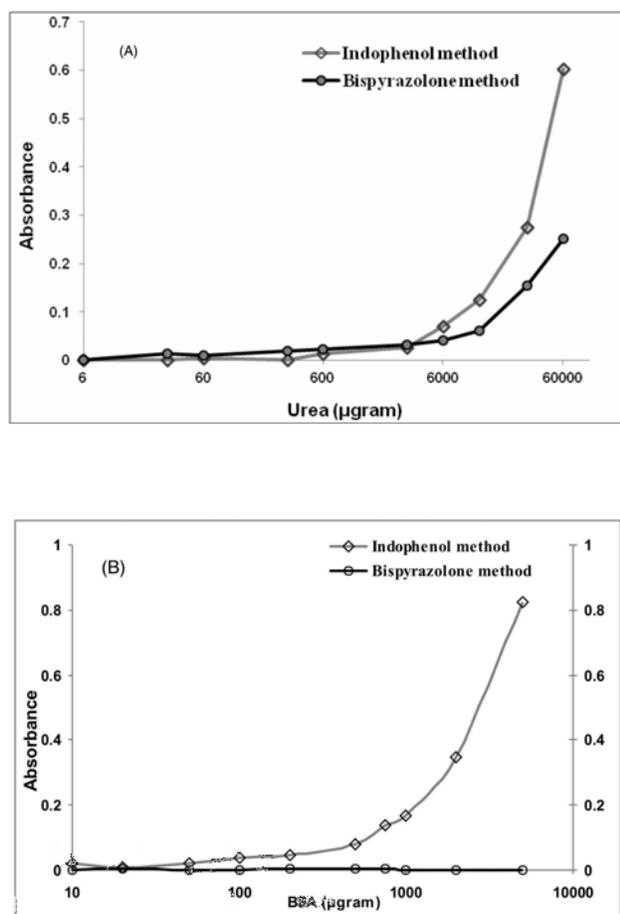


Fig. 4: Comparative evaluation of non-specific interferences by urea and protein in the indophenol (-o-), and bispyrazolone (-●-) methods. Data are mean of three determinations.

is comparatively less prone to non-specific interferences posed by urea and proteins. Besides improved operational time, broader workable range, and buffer compatibility, this bispyrazolone method does not use any corrosive reagent. This feature enables its automation in the future.

Finally, 15 human urine samples were incubated, each in triplicate, with urease and analyzed by the diacetyl monoxime, indophenol and bispyrazolone methods. Results are presented under Table 1. It is evident from Table 1 that the data obtained using the bispyrazolone method are satisfactory and in agreement with results obtained by the existing methods especially the DAM-TSC method.

Table 1: Results of urea estimation in different urine samples by three different methods. Data represents mean of three determinations for each sample

Urine sample	Indophenol method (mg/100 mL)	DAM-TSC (mg/100 mL)	Bispyrazolone method (mg/100 mL)
A.	1455	1457	1468
B.	1986	2027	1967
C.	556	576	576
D.	583	584	571
E.	1102	1128	1106
F.	923	962	978
G.	354	397	361
H.	1714	1637	1762
I.	586	620	579
J.	65	90	89
K.	542	636	586
L.	657	886	854
M.	582	883	842

4. Conclusion

In conclusion, the present bispyrazolone method reported here overcomes many of the disadvantages of previously published procedures for urea

quantitation. The present method uses non-corrosive reagents and the absorbance of the color complex obeys Beer's law over a wide range of urea concentrations. It does not require the use of elevated reaction temperatures or uncommon laboratory apparatus and shows significant immunity to interferences common to older assaying methods. It shows buffer compatibility and is amenable to automation and more specifically it is extremely rapid

(requiring less than 5 minutes to complete an analysis).

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