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The Effect of Potassium Hexacyanoferrate (II) Trihydrate in Eliminating Bilirubin Interference on Serum Uric Acid Estimation

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Abstract: *Serum uric acid estimation is of great importance as it is related to a wide range of health complications such as renal failure, cardiovascular diseases, type 2 diabetes, obstructive pulmonary diseases, hypertension and gout. Bilirubin interference on serum uric acid level leads to misinterpretation of the results, hence the use of an ideal reagent is very important. Datas are available on the inhibitory effect of ferricyanides up to a bilirubin concentration of 2mg/dL. Reagent based on uricase-peroxidase coupled reaction has been developed to minimize the interference effect of bilirubin. Potassium hexacyanoferrate (II) trihydrate is the key component in this reagent as it showed remarkable effect on reducing the interference effect. Using 0.925mg/L potassium hexacyanoferrate (II) trihydrate in reagent, inhibition up to 20mg/dL conjugated bilirubin and 30 mg/dL unconjugated bilirubin can be eliminated. In addition, we also included sodium dodecyl sulfate as an alternative to potassium hexacyanoferrate (II) trihydrate however no significant impact is observed.*

Keywords: *Uric acid; Hyperuricemia; Bilirubin interference; Potassium hexacyanoferrate (II) trihydrate; Uricase-peroxidase method.*

Abbreviations: *Uric acid – UA; Potassium hexacyanoferrate (II) trihydrate – PHT*

1. INTRODUCTION

Uric acid (UA) is the metabolic end product of heterocyclic aromatic organic compound, purine [1]. In 1776, UA was first isolated from the kidney stones by Carl Wilhelm Scheele. UA synthesis mainly takes place in the liver and in intestine as the final product of purine nucleotides (adenine and guanine) derived from animal proteins [2]. The normal reference range of UA in human blood is 3.6 to 7.4 mg/dL in men and 2.5 to 6.8 mg/dL in women. The solubility of UA in human blood is around 6.8 mg/dL which is close to the average concentration of UA. As humans lack the enzyme uricase to oxidize UA to more water soluble allantoin, excess UA forms deposits of monosodium urate crystals in the joints and urinary tracts leading to gout [3]. UA exhibits both anti-oxidant and pro-oxidant properties. When the level of UA is high (hyperuricemia) its pro-oxidant activity becomes prominent [4]. The anti-oxidant activity of UA is mainly through ferrum chelation [5] and can clear more than 60 % of free radicals in the body [1].

The antioxidant activity of UA is strongly linked with conditions such as myasthenia gravis,[6] acute ischemic stroke,[7] and neuromyelitis optica [8].Studies show that UA can protect the central nervous system by reducing the permeability of the blood brain barrier and also by inhibiting inflammatory cascades [9]. The pro-oxidant activity of UA takes part in the pathogenesis of Parkinson's disease [10].UA is also involved in the pathogenesis of depression by the inhibitory effect on peroxynitrite [11]. Hyperuricemia is associated with glucose metabolic disorders, [12] cardiovascular disorders, dyslipidemia, hypertension, [13,14] endothelial dysfunction and renal vasoconstriction [15]. Different studies have shown that UA is not only limited to the deposition of urate crystals but also to the pathogenesis of coronary heart disease, [16,17] chronic kidney disease, [18,19] and chronic obstructive pulmonary disease [20].

Serum UA can be quantitated by different methods. A colorimetric method is based on the reduction of phospho-tungstic acid by UA to produce a measurable color change of tungsten blue [21]. This technique is interfered by the presence of other reducing substances like ascorbic acid which can also reduce the phospho-tungstic acid. A high-performance liquid chromatography method on reversed phase columns accompanying either UV absorbance or mass spectrometry is also used [22,23]. The most common and widely accepted method is uricase-peroxidase coupled method [24,25]. In the present study we used the uricase-peroxidase method in which uricase converts UA into allantoin, with the formation of hydrogen peroxide. In presence of the enzyme peroxidase, nascent oxygen is generated which reacts with ethyl-sulphopropyl toluidine and 4-aminophenazone, to produce a violet colored complex. The intensity of this complex is measured at 548/660 nm wavelength and is directly proportional to the concentration of UA in the serum. Different studies are available on the interference effect of bilirubin in measuring serum UA by peroxidase coupled uricase method [26,27]. Bilirubin is the endproduct of heme metabolism [28]. High concentration of bilirubin inhibits the formation of reaction intermediate in the uricase-peroxidase method and thereby reducing the amount of colourd product [29].

The aim of this study is to develop a reagent to measure serum UA, without the interference of bilirubin. The role of potassium hexacyanoferrate (II) trihydrate (PHT) in minimizing the interference effect is considered. Several reports are available on the inhibitory effect of ferricyanideson the interference of bilirubin up to a concentration of 2 mg/dL [26,29].

2. Materials and Methods

2.1. Chemicals and Reagents

Bilirubin conjugate and free bilirubin were obtained from Lee Biosolutions. 4-amino antipyrine, enzymes uricase and peroxidase were purchased from Merck. Potassium dihydrogen phosphate and sodium hydroxide were procured from Sorachim. Methyl parahydroxy benzoate and PHT were acquired from Sigma. Stabilizers, preservatives and TOOS [N-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine] were obtained from Roche. We used Uric acid kit of Siemens Dimension Xpand Plus integrated chemistry systems as an external reference reagent.

2.2. Instruments

Siemens Dimension Xpand (Serial no: 2004081662), TBA 120FR (Serial no: 99A1612237) from Canon medical systems and Biolis 30i (Serial no: 6002221017) from Tokyo Boeki Medisys were used in this study.

2.3. Sample Preparation

High value UA samples were collected from Agappe staffs to obtain pooled sera. Written informed consent was obtained from all individuals included in the study. UA level on this pooled sample was measured by using commercially available kit of Siemens Dimension Xpand plus integrated chemistry systems and it was taken as control. To this control,50 mg/dL concentrations of both conjugated and unconjugated bilirubin (unconjugated bilirubin was dissolved in 0.1N sodium hydroxide solution) were added and it was further diluted with the pooled sera to get up to 5 mg/dL bilirubin concentration to study the interference effect. UA levels on the above samples were measured using the reagent with and without PHT.

3. Results And Discussion

Control sample with known value of UA was treated with different bilirubin concentrations (50 mg/dL, 40 mg/dL, 30 mg/dL, 20 mg/dL, 10 mg/dL and 5 mg/dL) and the level of UA in each treated sample was measured. Table 1 and 2 show the result of UA in different bilirubin treated sample, measured by using the reagent with and without PHT in two different fully auto analyzers. Results show that PHT can eliminate the interference effect of conjugated bilirubin up to 20 mg/dL concentration and unconjugated bilirubin up to a concentration of 30 mg/dL. This result contrasts with other researches [26,29] who observed that ferricyanides reduced the interference effect of bilirubin

hardly up to 2mg/dL concentration of bilirubin. Hence the present study indicated the significance of optimal concentration of PHT in effectively inhibiting high concentration of bilirubin. Fig 1 and 2 displays the amount of UA retained in control sample on the addition of various concentration of bilirubin as compared to control sample in terms of percentage of activity.

Table1. *The effect of PHT addition in the reagent for uric acid estimation in the control sample and treated samples withvarying concentrations of conjugated bilirubin.*

The concentration of uric acid in the control sample is 7.4 mg/dL.				
	Uric acid concentration (mg/dL)			
Conjugated bilirubin concentration in the treated sample (mg/dL)	TBA 120 FR		Biolis 30i	
	Reagent with PHT	Reagent without PHT	Reagent with PHT	Reagent without PHT
50	5.7	1.9	5.8	1.7
40	6.1	2.0	6.2	1.9
30	6.8	2.1	7.0	2.3
20	7.1	2.2	7.3	2.5
10	7.3	2.2	7.3	2.7
5	7.5	3.1	7.4	3.3

Table2. *The effect of PHT addition in the reagent for uric acid estimation in the control sample and treated samples with varying concentrations of unconjugated bilirubin.*

The concentration of uric acid in the control sample is 7.4 mg/dL.				
	Uric acid concentration (mg/dL)			
Unconjugated bilirubin concentration in the treated sample (mg/dL)	TBA 120 FR		Biolis 30i	
	Reagent with PHT	Reagent without PHT	Reagent with PHT	Reagent without PHT
50	6.5	2.7	6.8	3
40	6.6	2.9	7.2	3.1
30	7.1	3.0	7.3	3.1
20	7.4	3.1	7.3	3.4
10	7.3	3.4	7.3	3.7
5	7.5	3.9	7.4	4.2

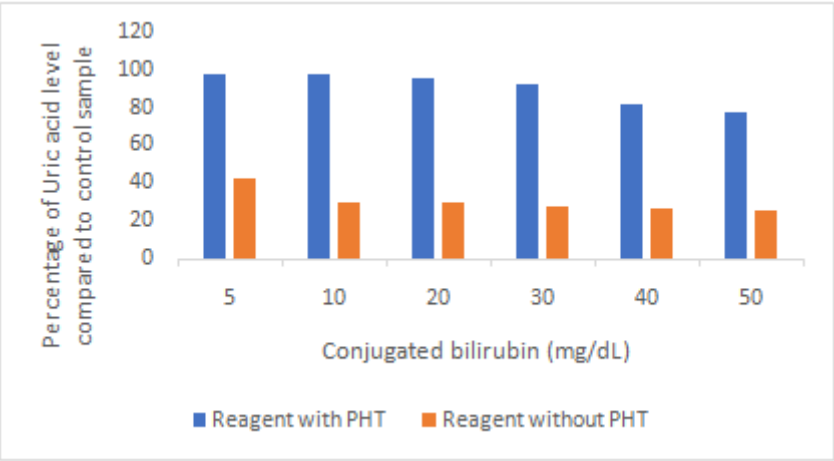

Figure1. *Percentage of uric acid level retained on the addition of conjugated bilirubin.*

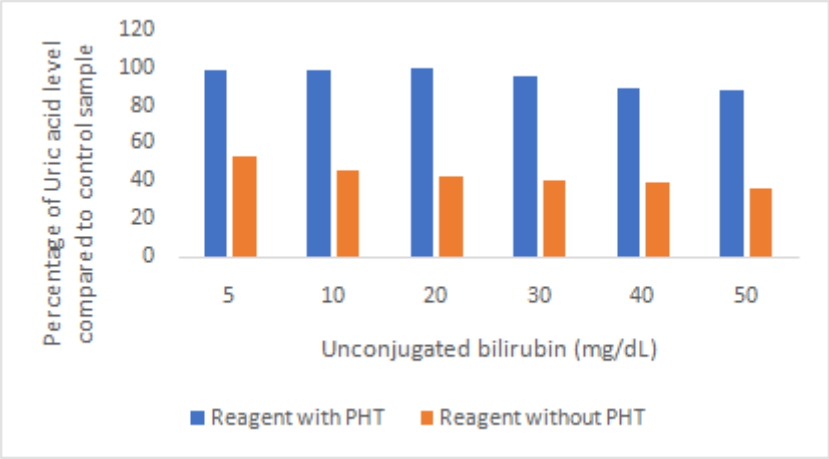

Figure2. *Percentage of uric acid level retained on the addition of unconjugated bilirubin.*

4. Conclusion

Bilirubin interference in UA estimation is a major issue which leads to misinterpretation of the results. In this work, bilirubin added sample is subjected to interact with reagent containing PHT. The result shows that PHT can completely inhibit the interference effect of conjugated bilirubin concentration up to 20 mg/dL and unconjugated bilirubin up to 30 mg/dL concentration.

5. Data Availability

Data sharing is not applicable to this research as no datasets were generated or analyzed during the present study.

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