Novel quantitative assay for estimation of ketone bodies in diabetic urine

E.S.Khan[$$], P.Karanjkar[++], V.N.Ravi Kishore.V†

Abstract-

Background: Diabetic ketoacidosis is a potentially life threatening acute disease which affects patients suffering from Diabetis Miletus. It leads to accumulation of ketone bodies namely acetoacetate [20%], β-hydroxybutyrate [78%] and acetone [2%] out of which acetone is formed in smaller quantities and readily been exhaled out of the body. Most of the ketone bodies estimation methods rely on estimation of acetoacetate and estimation of β- hydroxybutyrate is generally not done. We report a novel method for estimation of ketone bodies which estimate both acetoacetate and β- hydroxybutyrate together. Since β- hydroxybutyrate is formed in 78%, estimating both leads to enhanced sensitivity.
Method: The method is a spectrophotometric assay and it involved converting β- hydroxybutyrate to acetoacetate using Jones reagent and estimating the total acetoacetate by the existing methods. The conversion efficiency of this reaction was estimated by gas chromatography and the method was validated using IS HPLC performed simultaneously on diabetic urine samples.
Results: A standard curve was prepared with known concentrations of acetoacetate and it showed good linearity in the concentration range (0.2-11.76 mmol\L) at 335 nm. The method was successfully applied for estimation of ketone bodies in urine samples from diabetic patients and the results were consistent with those obtained using IS HPLC method used in clinical laboratories.
Conclusion: We developed & validated a sensitive, robust, cost-effective & convenient spectrophotometric assay for specific determination of ketone bodies [acetoacetate, β- hydroxybutyrate] in diabetic urine.

Index Terms— Ketoacidosis, Ketone bodies, Acetylacetone, Acetoacetate, β-hydroxybutyrate, Acetone, Diabetes Mellitus.

Abbreviations- HPLC-High Performance Liquid Chromatography, GC-MS – Gas Chromatography Mass Spectrophotometry, GC- Gas Chromatography, TLC- Thin Layer Liquid Chromatography , IS- Internal Standard, LC – Liquid Chromatography.

1 INTRODUCTION

In today's world Diabetic ketoacidosis is a major serious health disease due to the prevalence of increasing number of persons with diabetes mellitus [1, 2]. It is hypothesized that the number of individuals will double by 2025.Diabetes ketoacidosis was originally described by Dreshfeld in 1886 [2], until insulin was discovered. It is a life threatening condition that can occur when there is complete lack of insulin, for e.g. Type 1 Diabetes Mellitus, inadequate insulin levels associated with stress or even with Type 2 Diabetes Mellitus. Over the past 20 years, the number of diabetic ketoacidosis cases have increased from 3.4 % to 46 %. The frequency of this disease is high in United Arab Emirates, Saudi Arabia & Romania and lowest in Sweden, the Slovak Republic & Canada. [3, 4]. Also, in most Asian countries, Diabetic ketoacidosis has been found in children suffering from Diabetes & the adults in these countries have many other complications along with ketoacidosis such as myocardial infraction & other cardio vascular diseases [5, 6]. In 1997, it was reported that in Non-insulin dependent diabetes mellitus [NIDDM] young obese adolescent African-Americans the occurrence of ketosis was high between 1982 to 1995 and still it is increasing [7,11]. Therefore it is a demand today to control this disease before which diagnosis comes into account. Starvation, alcoholism, malnutrition & diabetes (hereditary disease) causes ketoacidosis [5, 15]. The frequency and clinical heterogeneity of diabetic ketoacidosis in a multiethnic population have significant implications for the diagnosis, classification and management of patients with Diabetes [10]. Diabetic ketoacidosis leads to accumulation of ketone bodies. There are three types of ketone bodies namely acetoacetate [20%], β-hydroxybutyrate [78%] and acetone [2%] out of which acetone is formed in smaller quantities and is readily exhaled out of the body. Generally acetoacetate and β-hydroxybutyrate are transported by the blood to the liver, where they are converted to acetyl CoA and oxidized in TCA cycle, providing energy required for the body [6]. In patients suffering from Diabetes, insulin levels are reduced and this reduction in insulin levels leads to increased production and accumulation of ketone bodies. In abnormal conditions the ketone bodies range are: Small: <20 mg/dL(< 1.96 mmol/L) , Large: >80 mg/dL (>7.84 mmol/L) [29,30]. The early symptoms of ketoacidosis are weakness of body, frequent thirst & urination, dry tongue and skin, leg cramps, fruity odor in breadth & upset stomach. Vomiting, shortness of breath, sunken eyeballs, rapid pulse and sudden stomach upset are the late signs and symptoms [9, 13].

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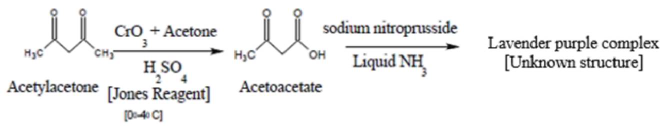

Fig No. 1.1

Chemical oxidation of Acetylacetone:

Acetyl acetone is converted into acetoacetate by jones reagent; further lavender purple complex is formed from acetoacetate reacting with sodium nitroprusside.

Latent Immune disorders [LID] can also cause diabetic ketoacidosis [8]. Serious complications include hypokalaemia/hyperkalemia, cerebral odema, hypoglycaemia & pulmonary oedema [13, 14]. In order to determine the presence of ketone bodies in blood and urine samples, a number of qualitative tests like Rothera and Gehardt tests are used [27]. Also Ketostrixs and Ascetest which are keto tablets [Ames Division of Miles Laboratories, Inc., Elkhart, Indiana] are designed to qualitatively analyze the samples. Enzymatic assays [24] and biosensor kits [Patent US-6541216] having enzymes in them have been designed which detect the changes in the enzyme induced reaction by amperometric methods. But the enzymes are expensive and the sensor kits are costly [18, 19, 20].Other sophisticated methods for quantitative estimations are HPLC and GC-MS which are expensive, tedious and time consuming [20, 21, 22, 25]. In this article, we describe a sensitive, reliable and cost effective standard protocol for the quantitative estimation of ketone bodies in urine of diabetic patients. This method was validated using the existing HPLC method of ketone bodies analysis. In this method the entire ketone bodies are converted to acetoacetate and is analyzed by formation of complex with sodium nitroprusside in presence of aqueous ammonia (Fig.No.1.4). A standard calibration curve was prepared by spectrophotometric assay. The basic principle of this assay was to estimate the total amount of ketone bodies by converting β-hydroxybutyrate into acetoacetate by chemical oxidation using Jones reagent (Fig.No.1.1).

Materials and Methods:

The standard curve for estimation of acetoacetate was prepared using Acetyl acetone [AR grade, A. B. Enterprises, India] in the concentration range 2.35 mmol to 11.76 mmol. Equal volumes of Jones reagent (prepared as given in [2]) and sodium nitroprusside (4 mg/mL) (AR grade, Sisco Research Laboratories Pvt. Ltd, India) were added as shown in Table No 1.1. This was followed by addition of 10 µL of chilled aqueous ammonia (AR grade, SD Fine Pvt. Limited, India) in all the vials (Table.No.1.1). The reaction was performed at low temperature (0 - 5^{0}C) as the acetoacetate formed in the reaction is highly unstable at room temperature [28]. This was followed by addition of D/W to make up the volume to 1 mL and the absorbance recorded at a wavelength of 335 nm within 3-5 mins.

The conversion efficiency of the reaction was estimated by gas chromatographic analysis. The GC system used consisted of RTx®5 column of 30m length, 0.25 mm. The samples used in GC were prepared in $CHCl_3$ and they consisted of Acetylacetone dissolved in $CHCl_3$ and the second sample consisted of unreacted Acetylacetone dissolved in $CHCl_3$ which was obtained by performing the reaction in $CHCl_3$/water mixture and separating the $CHCl_3$ layer from the water layer. Simultaneously, while performing the reaction the pH was monitored at each step. Also, TLC was performed using $CHCl_3$ as mobile phase and spot detection done using an iodine chamber.

In order to validate the new method of detection of ketone bodies in blood serum & urine, HPLC analysis was performed. The system consisted of an online degasser (DGU-14A), low-pressure gradient flow control valve (FCV-10ALvp), a solvent delivery pump (LC-10ATvp), an auto injector (SIL-10A), a column oven (CTO-Avp), a UV detector (SPD-20A), and a system controller (SCL-10Avp). Chromatographic separation was achieved on a Capcell Pak C_8 column (5 µm particle size, 150×4.6 mm i.d.), and the LC mobile phase consisted of 100% methanol. The column temperature was maintained at room temperature (25°C) during the entire separation process and the detection was carried out at 335nm with UV detector [12]. The mobile phase flow rate was maintained at 1mL/min with injection volume of 100µL of acetoacetate (11.76 mmol) prepared by our protocol as an Internal Standard. The diabetic urine samples of 10 patients were collected from Jairaj hospital, Navi mumbai (Sample A, C, F & I) & Metro Care Pathology Lab, Mumbai (Sample B, D, E, G & H). Diabetic urine were collected and stored at -80°C until the assay which was performed within 24 hours. The reaction mixture for HPLC were prepared by adding 1 µL of Jones reagent (prepared as given in [2]) at low temperature (0 - 5^{0}C) to the 1 µL of diabetic urine samples. This reaction mixture were directed to HPLC estimation under the same condition mentioned above for IS.Also, spectrophotometric absorbance at 335nm were recorded on same reaction mixture after addition of 1 µL sodium nitroprusside (4 mg/mL) (AR grade, Sisco Research Laboratories Pvt. Ltd, India), followed by addition of 10 µL of chilled aqueous ammonia (AR grade, SD Fine Pvt. Limited, India).

Results & Discussion:

A reliable and easy method is needed for determination of ketone bodies, especially in suspected cases of diabetic ketoacidosis. Harano [18] developed a highly sensitive and simplified spectrophotometric method using diazo reagent for determination of ketone bodies in blood. In our study, we designed a new method for spectrophotometric analysis of ketone bodies by converting all ketone bodies into acetoacetate using a chemical oxidation method. Our method offers the advantage of estimation of both β-hydroxybutyrate and acetoacetate simultaneously by converting β-hydroxybutyrate to acetoacetate which offers

Sr.no	Concentration of acetyl acetone (mmol/L)	Amount of acetyl acetone (μL)	Amount of jones reagent (μL)	Amount of sodium nitroprusside (μL)	Absorbance at 335 nm
Blank	0	0.2	0.2	0.2	0.0
1	2.35	0.2	0.2	0.2	0.184 ±0.001
2	4.70	0.4	0.4	0.4	0.500 ±0.025
3	7.05	0.6	0.6	0.6	0.750 ± 0.0375
4	9.41	0.8	0.8	0.8	0.950 ± 0.0475
5	11.76	1.0	1.0	1.0	1.19 ± 0.0595

Table No.1.1
Standard Curve Protocol for estimation of acetoacetate:
Standard dilution chart for estimation of acetoacetate using jones reagent as oxidant, sodium nitroprusside in presence of liquid ammonia as acetoacetate tracing agent.Spectrophotometric absorbance readings was measured at absorbance maxima at 335nm for developing standard protocol.

better sensitivity. In this assay, the acetylacetone is oxidized to form acetoacetate using Jones reagent. The resultant reaction mixture formed was immediately reacted with sodium nitroprusside in presence of aqueous ammonia to form lavender purple complex (Fig.No.1.1). The absorbance was recorded at 335nm and the standard calibration curve plotted. The curve showed good linearity (R^2 = 0.9955) in the concentration range 0.2-11.76 mmol\L with slope y = 0.1019 x as shown in Fig.No.1.2. The LOD (Lower limit of detection) of this assay is 0.2mmol\L. The HOD (Higher limit of detection) of this assay is 117. 6 mmol\L (Fig.No.1.2).

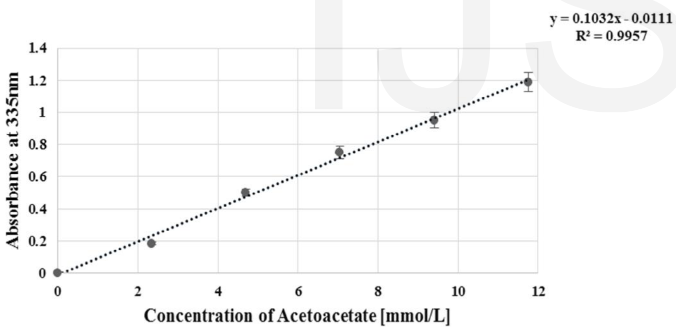

Fig No.1.2
Standard calibration curve:
Plot of absorbance vs concentration of acetoacetate is shown which is obtained by oxidation of acetylacetone with Jones Reagent and on further reaction with Sodium Nitroprusside under alkaline conditions. The absorbance being measured at 335 nm.

The conversion efficiency of the reaction was estimated by gas chromatographic analysis. About 92.76% of product was formed as shown in [Fig.No.1.3 & Table No.1.2] at a retention time of 2.9 mins. Absorbance spectrum of the same samples indicated that reactant is 90 % less in the product as shown in Fig.No.1.6. This suggests that oxidation reaction was successfully performed yielding desired product. Also, pH was monitored at every step of reaction indicating change in pH from 6 to 1 as the reaction was performed. The TLC experiment supported the results of GC analysis suggesting approx. 90% of reactant i.e. acetylacetone into acetoacetate (product).

	Reactant (Acetyl acetone) (mmol/L)	**Product (Acetoacetate) (mmol/L)**
Concentration	4.70 X 10^{-3}	4.36 X 10^{-3}
Retention time (sec)	2.966	2.906
Peak height (a.u.)	3355725	1230149
Area under curve	16622245	1230149
Yield	--	92.76% Product
Mobile phase	Methanol (100%)	Methanol (100%)

Table No 1.2:
Estimation of the percentage conversion of acetylacetone to acetoacetate by gas chromatography analysis
This table depicts the estimation of conversion efficacy of reactant (Acetylacetone) to product (Acetoacetate) using gas chromatography. As shown in the fig 1.4. About 92.76% of product was formed as shown above at a retention time of 2.9 mins. This suggests that the oxidation reaction was successfully performed yielding the desired product.

The optimized assay was completely validated using HPLC estimations on diabetic urine samples according to the guidelines in ICH guidelines Q2 (R1) for validation of analytical methods. The reproducible results were obtained using new assay reported here & HPLC method of estimation from the IS & urine samples. According to the abnormal condition reference range mentioned above the Sample A, G & H had moderate to large amount of ketone bodies, Sample B & I had moderate levels of ketones bodies & Sample F had small amount of ketone bodies. We suggest that all this Diabetic patients are suffering from ketoacidosis. Sample C, D & E had normal ketone bodies count suggesting that this people did not suffer from the disease. It appears that the values obtained by the HPLC method tend to be slightly low compared with those obtained by current new method. [Table 1.3]. This method is unique, robust, reliable, cost effective and easy as compared to other sophisticated methods.

Sample	Patient no:	Sample	Amount of ketone bodies [HPLC Method] [mmol/L] Retention Time : 0.671 min	Amount of ketone bodies [UV Spec Method] [mmol/L]	Condition
A	C15070490	Urine	4.743	4.80	**Moderate to Large***
B	LC020F435	Urine	2.961	3.10	**Moderate***
C	C18001351	Urine	0.687	0.68	**Normal**
D	LC0934155	Urine	0.944	1	**Normal**
E	LC0981161	Urine	0.768	0.77	**Normal**
F	C18001475	Urine	1.954	2	**Small***
G	LC0361439	Urine	6.023	6.16	**Moderate to Large***
H	LC0361440	Urine	5.731	5.78	**Moderate to Large***
I	C18049701	Urine	3.64	3.8	**Moderate***

Table 1.3:
Validation of our assay by IS HPLC method
Amount of ketone bodies measured in mmol/L by HPLC and Assay in urine samples of patients with an indication for ketoacidosis condition. It seems that the values obtained by the HPLC method tend to be slightly low compared with those obtained by new assay.

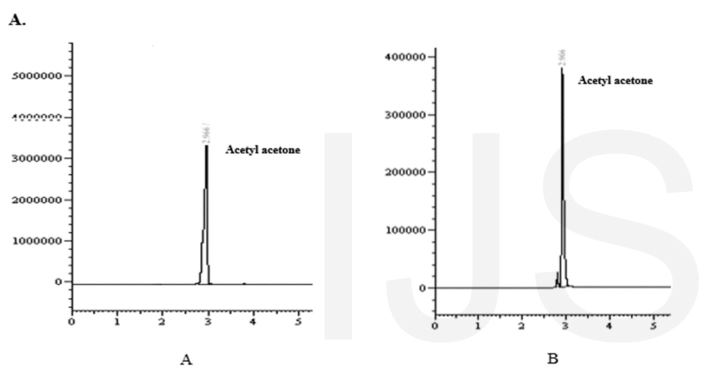

Figure No.1.3.
Estimation of conversion efficacy of reactant (Acetylacetone) to product (Acetoacetate) using gas chromatography
A: 100% Reactant (Acetyl acetone) B: Unreacted Reactant (Acetyl acetone)

The uniqueness of this assay is the catalytic oxidation of β-Hydroxybutyrate into acetoacetate. The prime advantage of this method is its greater sensitivity & convenience. Multiple estimations can be carried out within few minutes, the time taken is rather less than other methods. This method can be used as an important method for accurate quantification of ketone bodies in urine. The acetoacetate values obtained by the new method are in agreement with IS HPLC method along with greater specificity as mentioned above which signify its novelty. Using this assay cost effective biosensor can be designed in future which can aid in handy analysis of ketone bodies.

Conclusion:

We have established a new spectrometric assay for determination of two ketone bodies, acetoacetate & β-hydroxybutyrate, in urine of diabetic patients. This method was directly compared to IS HPLC method at 335 nm. Total ketone bodies in urine were determined by the complete conversion of β-hydroxybutyrate to acetoacetate in presence of chemical oxidation reagent i.e. Jones reagent. The proposed method has the advantage of using a small sample volume (0.2 µL) and is very sensitive and reliable within a broad range from 0.2 mmol / L to 11.76 mmol / L of ketone bodies for determination of acetoacetate and total ketone bodies. It is also a well-validated procedure. Furthermore, this new method shows excellent interrelationship in comparison with the IS HPLC method used in clinical chemistry laboratories.

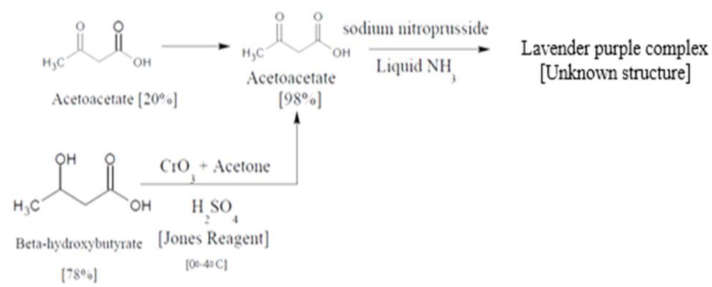

Fig. 1.4
Conversion of all ketone bodies into acetoacetate using Jones Reagent and its estimation using sodium nitroprusside under alkaline conditions
β-hydroxybutyrate is converted to acetoacetate using Jones reagent at 0-4 ° C. β-hydroxybutyrate along with acetoacetate constitutes total of 98% of ketone bodies. This conversion to acetoacetate is followed by reaction with sodium nitroprusside in presence of aqueous ammonia which finally gives a lavender-purple complex.

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