Novel quantitative assay for estimation of ketone bodies in diabetic urine

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Abstract

Background: Diabetic ketoacidosis is a potentially life threatening acute disease which affects patients suffering from Diabetes Mellitus. 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 & 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 355 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— Ketoadicosis, Ketone bodies, Acetylacetone, Acetoacetate, β-hydroxybutyrate, Acetone, Diabetes Mellitus.

Latent Immune disorders [LID] can also cause diabetic ketoacidosis [8]. Serious complications include hypokalemia/ hyperkalemia, cerebral oedema, 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 Acetetylacetone [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°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® column of 30m length, 0.25 mm. The samples used in GC were prepared in CHCl₃ and they consisted of Acetylacetone dissolved in CHCl₃ and the second sample consisted of unreacted Acetylacetone dissolved in CHCl₃ which was obtained by performing the reaction in CHCl₃/ water mixture and separating the CHCl₃ layer from the water layer. Simultaneously, while performing the reaction the pH was monitored at each step. Also, TLC was performed using CHCl₃ 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-10A), 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-10A/vp). Chromatographic separation was achieved on a Capcell Pak C₈ column (5 µm particle size, 150x4.6 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°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
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\textperml 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\textperml. The HOD (Higher limit of detection) of this assay is 117.6 mmol\textperml.

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. The TLC experiment supported the results of GC analysis suggesting approx. 90% of reactant i.e. acetylacetone into acetoacetate (product).

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

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.

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 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.
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 \(\beta\)-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 & \(\beta\)-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 \(\beta\)-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.

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.

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

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References:


