A novel chemical method for measuring ketone bodies in subclinical ketosis

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Abstract:

BACKGROUNDS: Subclinical ketosis can cause greater economic loss due to a lack of clinical symptoms. **OBJECTIVES:** The present study was aimed to design a chemical method for measuring serum ketone bodies in the affected subclinical cows. **METHODS:** Acetoacetate concentrations were measured using a nitroprusside reaction and β-hydroxy butyric acid (BHBA) which was oxidized to acetoacetate using nanosilver particles to determine its concentration. Recovery tests were done for different concentrations of betahydroxy butyrate in bovine pooled serum. **RESULTS:** Actoacetate levels were in range of 0.1-6 mM, and the values for BHBA were found to be in the range of 0.125-3 mM. **CONCLUSIONS:** It was concluded that this technique can be considered as a simple method for measuring ketone bodies in biological fluids.

Introduction

Quantification of blood lactate, pyruvate, β-hydroxybutyrate and acetoacetate is crucial in diagnosing and monitoring metabolic acidosis. In this respect, measurement of β-hydroxybutyrate and acetoacetate can be helpful in differential diagnosis and the monitoring of ketoacidosis (Ingvartsen, 2006).

Fatty liver syndrome and diabetic ketoacidosis are widespread diseases among different animal species. In both cases there are increased levels of ketone bodies. Measuring ketone bodies, especially β-hydroxybutyrate, can be useful in clinical management of subclinical ketoacidosis (Seung-Kwon and Chanhee, 2010).

Economic loss due to subclinical ketoacidosis is a major problem in industrial animal husbandry. A disorder in the lipid metabolism produces a lot of β-hydroxy β-methyl glutaryl CoA, which will be converted to acetoacetate and acetone. Consequently, acetoacetate, an intermediate compound, will be converted to β-hydroxybutyrate (Nocek, 1997), and there is a direct relationship between the levels of β-hydroxybutyrate and economic loss in industrial bovine farms.

There are different methods that have been established for measuring blood acetoacetate and acetone levels. In this respect, several methods have been proposed for the measuring of β-hydroxybutyrate levels. β-hydroxybutyrate will be oxidized and turn into acetoacetate. The consequence of this is that the dye is bound producing a chromophore (Galan et al., 2001).

Different chemical (Zaidi, 1993), and enzymatic (Galan, 2001), methods were used for oxidizing β-hydroxybutyrate to create acetoacetate. Chemical methods are too laborious, time consuming and less sensitive. On the other hand, enzymatic methods are too time consuming (Zaidi, 1993). In this respect, establishing an efficient, inexpensive method can be useful for clinical use. Previously, a combination of

AgNo3 and K2S2O8 has been used for converting BHBA to acetoacetate. However, stability of the chromophor compound was too low to be used as a clinical method (Zaidi, 1993). The aim of the present study was to produce a stable chromophore through the converting of BHBA to ACAC by means of combining Natrium metaperiodate and Nanosilver through a spectrophotometric method.

Materials and Methods

Acetoacetate measuring: With some modifications, acetoacetate levels were measured by the Schilk and Jhonson,1965. In brief, we prepared acetoacetate (Sigma, A8509) standard solutions (1,2,3,4,5,6,7,8, 9,10 mM) in an aminoacetic acid (Merck, 548955137 6) buffer. Then, Natrium Nitroprosside (Merck, 6540) solution was added and incubated for 18 minutes at room temperature. Absorbance was read at 550 nm, against the control blank.

Determination of hemoglobin interactions: Hemoglobin standard solution (Zist Cimi Chemical Company, 30 mg/dL) was prepared and added to different concentrations of acetoacetate solution. Absorbances of these solutions were compared with solutions without hemoglobin to find the hemoglobin interactive effects on the absorbance.

BHBA measuring: BHBA was oxidized to acetoacetate by means of sodium metaperiodate (Merck, 6597) in the presence of nanosilver solution (Nanosilver, pore size <20 nm; concentration 8000 ppm, MEG base, NCC Engineering Company, Tehran, Iran). The resulting acetoacetate levels were measured as mentioned above.

Color development and its stability: 3mM BHBA solution was put in a cuvette and its absorbance was monitored for 30 minutes to follow the colored complex stability.

Effect of pH on the reaction: The effect of acidic, alkaline and neutral conditions on the color development was studied and the reactions were performed at pH 5.2, 7 and 8.6 to determine the linearity of the concentrations vs. absorbance.

Analytical recovery and precision: For recovery and precision studies, we added defined levels of BHBA to both buffer and sera for determining BHBA levels in both buffer and serum media. In this respect, serum was deproteinized by the modified Foli-Wu method (Glick, 1959). In this respect, blood was collected from the clinically healthy cows (Dashte Javid Farm, Tehran, Iran). Serum was separated after centrifugation and protein deproteinization was done using Sodium tungestate 10% and sulfuric acid. BHBA values were determined by both enzymatic and chemical methods.

Statistical analysis: Slope values of the linear regression lines were compared using a upaired t-test by means of SigmaStat 2.0 (Systat Software Inc., Point Richmond, CA, USA). Alpha in all cases was 0.05 ($p<0.05$).

Results

Acetoacetate levels showed a complete linear correlation (r2= 0.9985) with absorbance in the range of 0.1- 6 mM (Figures 1 and 2). Total run-to-run and day-to-day CVs for acetoacetate levels were about 2.84% and 3.48%. In the recovery tests, acetoacete values showed a complete recovery in the range of 0.1-6 mM (Figures 3 and 4). While acidic and neutral conditions make some adverse effect on the stability of the colored complex, the alkaline pH was the most favorable one in terms of colored complex stability (Figures 5, 6 and 7). Invisible hemolysis (30 mg/dL) had no side effect on the linearity of the acetoacetate concentrations vs. absorbance in the range of 0.1-6 mM (Figure 8).

tests for measuring BHBA levels in the buffer showed a linear correlation in the range of 0.125-3 mM (Figures 9a, 9b). On the other hand, recovery of the BHBA levels from the pooled bovine serum, when measured, showed more than 92% for the chemical, and 94% for the enzymatic methods. Table1 shows added concentrations of BHBA to the pooled serum and the calculated values by chemical and enzymatic methods (BHBA recovery values).

Discussion

The present findings on acetoacetate concentrations vs. absorbance show a strong agreement with Zaidi's (1993) findings when using the chemical method. We have shown a linear relationship between acetoacetate concentrations and absorbance over a wide range of acetoacetate concentrations. It has been previously discussed that aminoacetic acid forms an intermediate

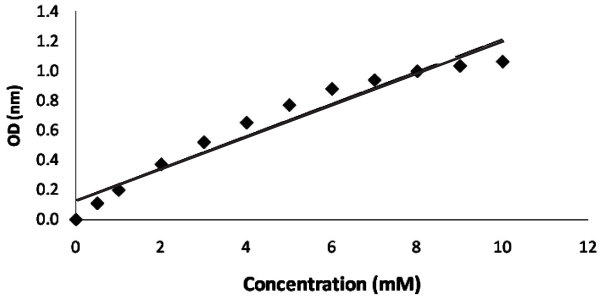

Figure 1. The relationship between absorbance and acetoacetate concentrations in the range of 0-10mM (r2 =0.9456). Acetoacetic STD curve. Y= 0.1086x+1274. R^2=0.9456.

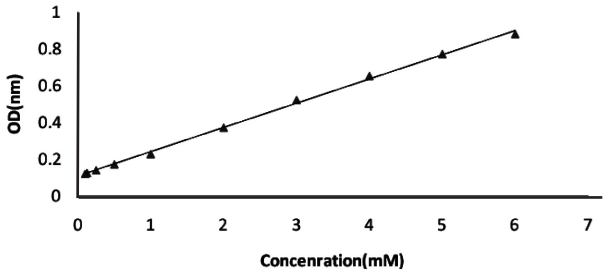

Figure 2. The relationship between absorbance and acetoacetate concentrations in the range of 0-6mM (r2 =0.9985). Acetoacetate STD curve. Y=0.132x+ 0.1092. R^2=0.9985.

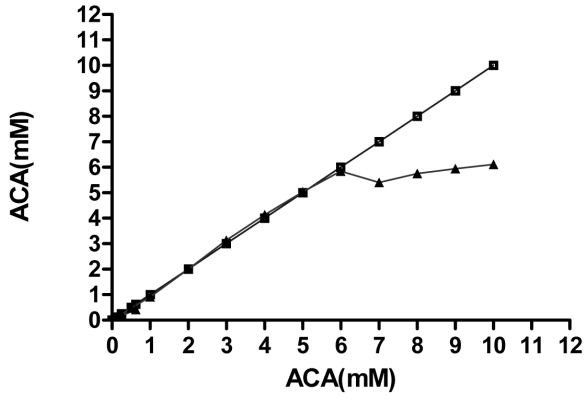

Figure 3. Linear regression line acetoacetate recovery in the range of 0-10 mM. —■— Actual Value —▲— Measured Value

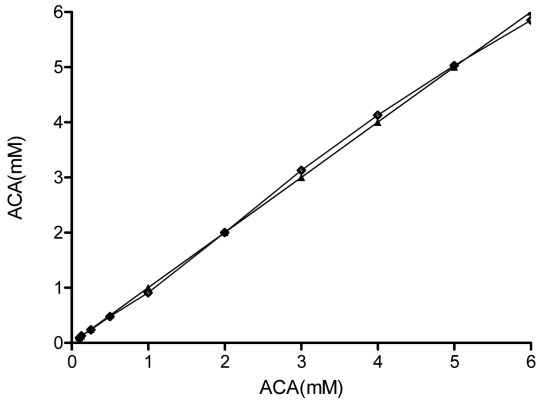

Figure 4. Linear regression line of actoacetate recovery in the range of 0-6 mM. —▲— Actual Value —◆— Measured Value

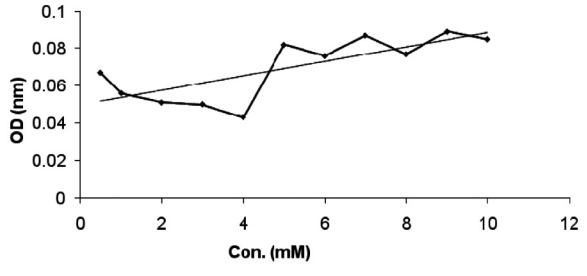

Figure 5. The effect of acidic condition (pH=5.2) on the reaction stability. Y=0.0039x+0.0499. R^2=0.56.

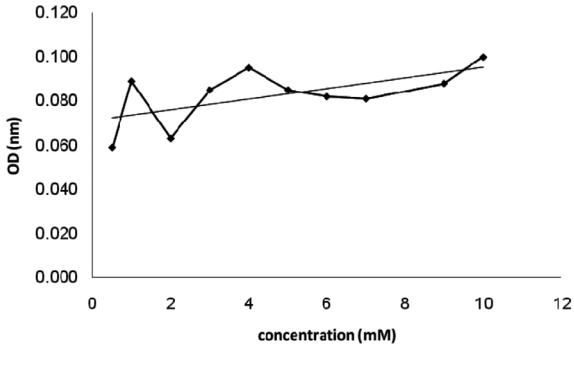

Figure 6. The effect of neutral condition (pH=7) on the reaction stability. Y=0.0024x+0.0714. R^2=0.3644.

compound with the acetoacetic acid which produces a colored complex with Sodium nitroprusside.

Studies on pH showed that an alkaline solution would be a suitable condition for complex formation and stability. Fischer et al, 1944 discussed aminoacetic acid forming a pyrrol when in this condition. Our

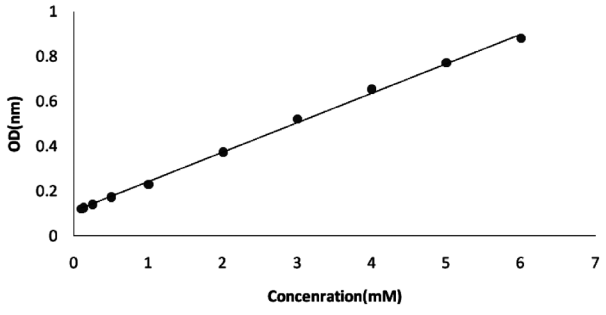

Figure 7. The effect of alkaline condition(pH=8.6) on the reaction stability. Y=0.132x+0.1092. R^2=0.9985.

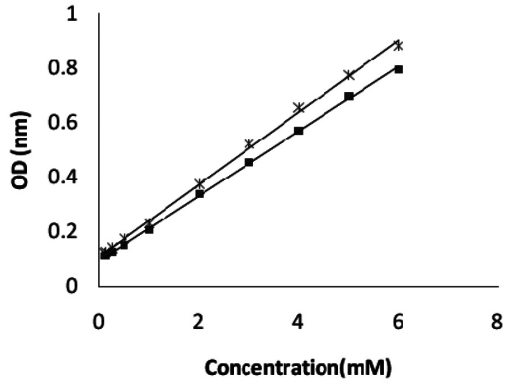

Figure 8. Linear regression lines of acetoacetate measuring in the presence and absence of hemoglobin.
Y=0.132x+0.1092. R^2=0.9985.
Y=0.118x+0.0959. R^2=0.9994.
✱ Basal state (whithout Lysate) ■ After adding Lysate

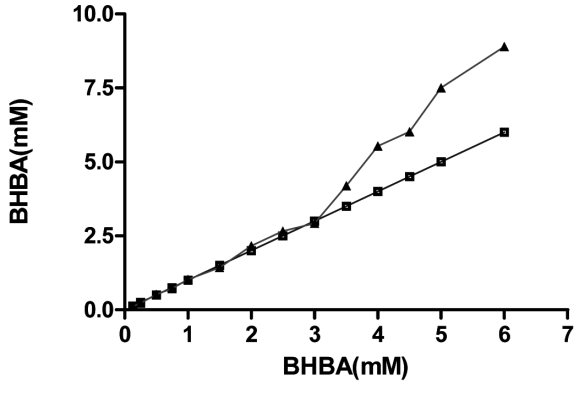

Figure 9a. The values of recovery for BHBA.
Actual Value Measured Value

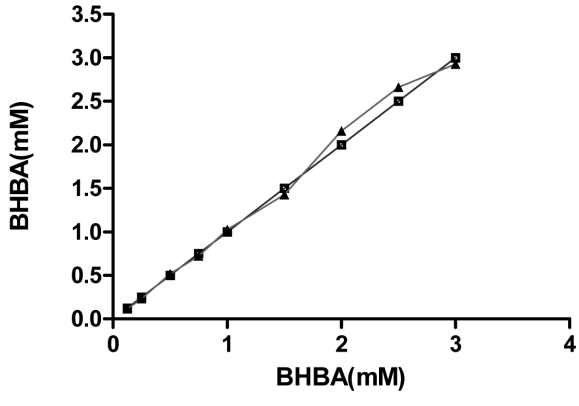

Figure 9b. The values of recovery for BHBA.
Actual Value Measured Value

Table 1. Comparison of Error percent for BHBA recovery by means of chemical and enzymatic methods.

BHBA Concentration (mM)	Error in the chemical method(%)	Error in the enzymatic method(%)
0.1	0	4.76
0.2	0	0.53
0.4	0	5.82
0.8	0	2.12
1	0	0.53
1.3	0	2.32
1.5	0	0.18
2	4.64	4.76
2.5	5.86	0.32
3	5.39	1.59
3.2	0	0.86
3.5	0.42	14.89
4	4.51	0

findings on pH showed that conversion of aminoacetic acid could be facilitated in the alkaline condition to produce a more stable colored complex.

We have shown a good recovery for BHBA from both the buffer and pooled sera. However, recovery showed some decline in the case of pooled serum. It seems that some compounds may interfere with colored complex formation. In this respect, Zaidi, 1993 showed that the chloride ion can interfere with the silver catalyst function which increases the nanosilver levels in the reaction mixture. Chloride precipitation may be a useful method for increasing recovery.

Blood BHBA evaluation is a better indicator of metabolic control, compared to urine ketone bodies detection, and is useful to predict the time required for

blood ketone bodies to clear (Prisco et al., 2006). On the other hand, serum ketone and blood BHBA measurements are equally effective in diagnosing diabetic keyoacidosis among uncomplicated cases (Tantiwong et al., 2005).

In conclusion, we presented a simple and specific colorimetric method for quantitative determination of acetoacetate and BHBA in body fluids.

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روش شیمیایی نوین برای اندازه گیری اجسام کتونی سرم در شرایط کتوز تحت بالینی گاو

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چکیده

زمینه مطالعه: کتوز تحت بالینی بعلت فقدان علائم بالینی می تواند منجر به ضرر اقتصادی هنگفتی در سطح گله شود. **هدف:** هدف از مطالعه حاضر طراحی یک روش شیمیایی جهت اندازه گیری غلظت اجسام کتونی در گاوهای مبتلا به کتوز تحت بالینی است. **روش کار:** غلظت استواستات با استفاده از واکنش نیتروپروکساید تعیین گردید همچنین بتاهیدروکسی بوتیرات (BHBA) در ابتدا توسط ذرات نانو نقره به استواستات اکسید گردید و در ادامه غلظت آن تعیین گردید تست های بازبینی با استفاده از غلظت های مختلف بتاهیدروکسی بوتیرات در سرم pooled انجام گردید. **نتایج:** غلظت استواستات در دامنه ۰/۶mM-۰/۱ و غلظت BAHA در محدوده ۳mM-۰/۱۲۵ تعیین گردید. **نتیجه گیری نهایی:** می توان نتیجه گرفت که این تکنیک می تواند بعنوان یک روش ساده جهت اندازه گیری اجسام کتونی در مایعات بیولوژیک مورد استفاده قرار گیرد.

واژه های کلیدی: کتوز، بتاهیدروکسی بوتیرات، استواستات، نانونقره .

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