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EXPERIMENTAL RESEARCH

Development Of A Solid Phase Single Reagent For The Detection Of Ketone Bodies

PALANDURKAR K * , BASAK A **

ABSTRACT

Introduction: Ketone bodies are found in an increased quantity in Diabetic ketoacidosis (DKA) and in Starvation and Alcohol induced ketoacidosis. The estimation of ketone bodies is of prime importance for the proper management of these conditions. We had developed the formulation of a solid phase single reagent for the detection of ketone bodies in urine / plasma so that anyone who was interested could prepare the reagent on their own, thus, adding to the cost cutting of investigation and self- reliance.

Aim: To develop a sensitive solid phase single reagent for the detection of ketone bodies in urine, to replace the commonly done, multiple step Rothera's test.

Materials And Methods: Sodium nitroprusside, glycine, disodium hydrogen phosphate and lactose for preparation of the reagent and acetone solutions of different concentrations for testing.

Results: The final composition of the reagent is as follows:
The maximum colour development was found with a homogeneous powder of Glycine: 2.5gm, Lactose: 1gm, Disodium hydrogen phosphate: 4 gm and Sodium nitroprusside: 400mg. It gives lavender to dark purple colour with increasing concentrations of acetone. The lowest concentration detectable by this reagent was found to be 5mg/dL of acetone.

Discussion: This reagent detects acetone semi-quantitatively and acetoacetate in plasma, but not β-hydroxybutyrate. This is helpful to the patient in ketoacidosis. This preparation can also differentiate between the plasma of a normal person and a DKA patient.

Conclusion: A sensitive solid phase single reagent for the detection of ketone bodies has been successfully developed.

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Introduction

Ketone bodies are the products of excessive fat catabolism and are produced in Type 1 Diabetes mellitus and in Starvation and Alcohol induced ketoacidosis due to the impairment of the Krebs cycle [1].

Their estimation is of prime importance for the proper management of DKA. Rothera's test [2] is done for the detection of ketone bodies but it is a multi-step, time-consuming and low sensitivity procedure. For overcoming these problems, a single reagent has already been developed in tablet form (Acetest) [2]. One drop of urine is to be dropped on the tablet and a lavender-purple colour develops quickly. The intensity of the colour gives the semi-quantitative estimation of the ketone bodies. The ingredients and the composition of the Acetest are the secret of the company. Only a few companies in India (Span Diagnostics, Pathozyme etc.) have developed the reagent in powder form

and this is available in the market. Some other companies have come up with dip-stick estimation (Ketosticks)[2]. We came up with a solid phase single reagent for the detection of ketone bodies in urine, so that anyone who is interested can prepare the reagent on their own, thus adding to the cost cutting of the investigation and self-reliance.

Aim

To develop a sensitive solid phase single reagent for the detection of ketone bodies in urine, to replace the commonly done, multiple step Rothera's test.

Materials And Methods:

Principle Of The Test

Acetoacetate or Acetone in presence of glycine, reacts with sodium nitroprusside to form a lavender-purple coloured complex. Disodium hydrogen phosphate provides the optimum pH for the reaction and lactose enhances the colour (3).

Reagents

Glycine, Lactose, Disodium hydrogen phosphate, Sodium nitroprusside and Acetone: All the reagents used for this experiment were of analytical grade.

Equipments

Mortar and Pestle, pH meter (ELICO), Electronic balance (Ohaus, USA), Slides / test tubes and Micropipettes

Preparation Of Acetone Standards

Acetone (sp. gr. 0.792) was diluted with analytical water to prepare standards of the concentrations: 5, 10, 20, 30, 50, 75,100,150 and 200mg/dL.

Preparation of a solid phase single reagent for the detection of ketone bodies

This work was started initially with 500mg Glycine, 100 mg Sodium nitroprusside, 2gm Disodium hydrogen phosphate and 200mg Lactose – the quantities of each chemical were empirical. All these reagents were mixed and ground together in a mortar to make a uniform fine powder. Special precaution was taken so that the red coloured Sodium nitroprusside crystals were finely powdered.

Testing Of The Solid Phase Single Reagent

A pinch of the above prepared powder was taken on a glass slide, with a white paper background. Then, two drops of 20mg/dL Acetone standard was mixed with the powder and this was observed for colour development.

Choosing The Best Composition For The Reagent

A series of experiments were carried out with the reagents. The strategy we used was: to keep three chemicals in fixed amounts and to prepare a series of combinations by gradual increment of the amount of the fourth chemical. All these reagents which were prepared were then simultaneously tested in series to find out which combination worked the best. Then, the best composition of that chemical was kept fixed for subsequent experiments. This process was repeated as described above for the remaining chemicals, one by one. The whole process was repeated twice for the formulation of the final reagent.

Results

The composition of the final reagent that we found was as follows:

Maximum colour development was found with

1) Glycine: 2.5gm [Table/Fig 1] (Fig. 1-a and -b)
2) Lactose: 1gm [Table/Fig 2] (Fig. 2-a and -b)
3) Disodium hydrogen phosphate: 4 gm [Table/Fig 3] (Fig. 3-a and -b)
4) Sodium nitroprusside: With increasing amounts, the colour intensity increased gradually. The maximum colour

development was found with 800mg [Table/Fig 4] (Fig. 4-a). But, with increasing amounts, the blank's colour also increased, making differentiation at the lower concentrations of acetone difficult. This was fixed to 400mg [Table/Fig 4] (Fig. 4-b) for good colour differentiation of the test from the blank.

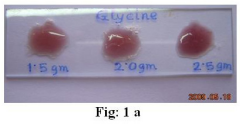


Fig: 1 a

Fig: 1 b

(Table/Fig 1)

Fig: 2- a

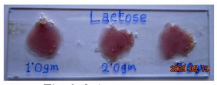


Fig: 2- b-1

Fig: 2- b-2 Dried slide

(Table/Fig 2)

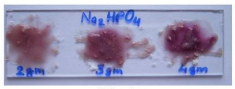


Fig: 3-a

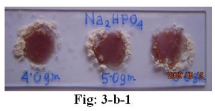


Fig: 3-b-1

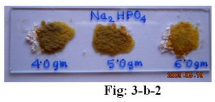


Fig: 3-b-2

(Table/Fig 3)

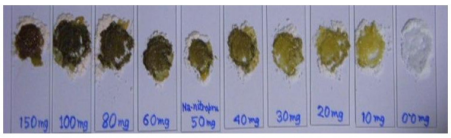


Fig: 4-a (amount ×10)

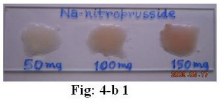


Fig: 4-b 1

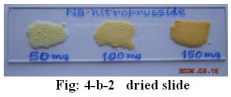


Fig: 4-b-2 dried slide

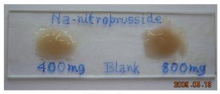


Fig: 4-c

(Table/Fig 4)

pH of the reagent

This reaction is pH dependent and works in alkaline pH. For taking pH, the solid phase reagent was dissolved in 5ml of water to get a saturated solution. The pH was found to be 8.26.

Range Of Detection

With increasing concentrations of acetone solutions from 5-200 mg/dL, the colour intensity increased accordingly [Table/Fig 5] (Fig: 5-a, b, c and d). The lowest detectable concentration was 5mg/dL.

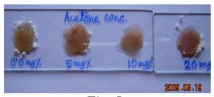


Fig: 5-a

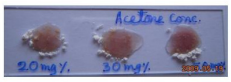


Fig: 5-b

Fig: 5-c

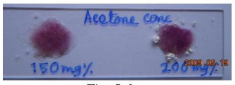


Fig: 5-d

(Table/Fig 5)

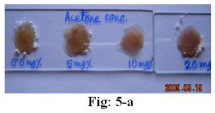


Fig: 5-a

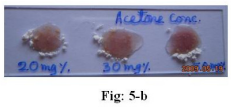


Fig: 5-b

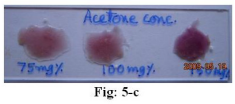


Fig: 5-c

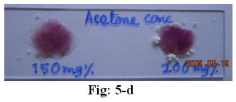


Fig: 5-d

(Table/Fig 5)

Discussion

We started this work based on the Principle of reaction [3]. While searching for the references, we found that very less information was available and that no composition of the reagents was found. So as to formulate the best composition, we started with one empirical composition mentioned in materials and methods. It did show change of colour to lavender-purple with acetone solution. So, we proceeded to formulate the best combination.

To sort this out, we developed the strategy of fixing three out of four chemicals and varying the remaining fourth one, starting from the lowest possible concentration to higher amounts. The amount of the chemical in the reagent which gave the maximum colour intensity was fixed and then, the remaining chemicals were tried in the same manner. In this way, the combination of the chemicals which gave the maximum colour

intensity was fixed. This was repeated for two cycles to develop a sensitive formulation.

1) Glycine: This is an important constituent of the reagent which takes part in the reaction. On gradual increment of it's concentration from 0.5 gm to 2.5 gm, the colour intensity increased progressively, but after that, the colour intensity development gradually dropped down. This effect might be due to the buffering action of glycine. The optimum pH of the reaction was 8.26, which was lowered by addition of excess amounts of glycine. So, after these experiments, the concentration of glycine was fixed to 2.5gm [Table/Fig 1] (Fig. 1-a and -b).

2) Lactose: It is added to the reagent to increase the colour intensity. After a few experiments, we found that above 1gm, the colour intensity decreased progressively. So we fixed it to 1gm [Table/Fig 2]. (Fig. 2-a, -b and -c)

3) Disodium hydrogen phosphate: This chemical of the reagent does not take part directly in the reaction, but it is required to maintain the optimum alkaline pH of the reaction. It was started with 2 gm and then increments of 1gm were made to find out the concentration which was required for maximum colour development. The reagent with 4gm of this chemical showed maximum colour development and any further increments decreased the colour intensity [Table/Fig 3] (fig 3-a, -b & -c).The pH which was maintained by 4gms of disodium hydrogen phosphate was 8.26.

4) Sodium nitroprusside: It was observed that the colour of the reagent increased as we went on increasing the amount of this chemical from 0.0 gm to 1.5 gm [Table/Fig 4] (Fig. 4-a). But the lavender-purple colour remained till 400mg of sodium nitroprusside and after that, the colour gradually started changing to reddish brown. We also observed that with high sodium nitroprusside amounts the colour of the blank started becoming reddish and at lower concentrations of acetone, it became difficult to distinguish the positive test result from that of the blank. So, we fixed the amount to 400 mg, which gave good differentiation between the test and the blank with 5mg/dL of acetone solution [Table/Fig 4](Fig. 4 -b and -c)

The total weight of the reaction mixture was 7.9gm.

It was then reacted with increasing concentrations of standard acetone solutions ranging from 5 to 200mg/dL. This showed a gradual increment in the colour intensity[Table/Fig 5] (Fig. 5-a, -b, -c and -d). Thus, it became ready to be used on patient samples for confirmation. A DKA patient's urine gave good colour differentiation as compared to that of a healthy patient's urine [Table/Fig 7] (Fig.7). This reagent is also useful for the detection of ketone bodies in plasma [Table/Fig 7] (Fig.7). There was more intense colour in plasma as compared to the urine of the same patient [Table/Fig 7] (Fig.7). This is because plasma is a direct indicator of the internal environment of body, while in urine, the excretory products are diluted.

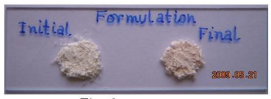


Fig: 6-a

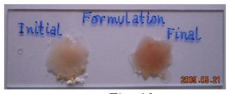
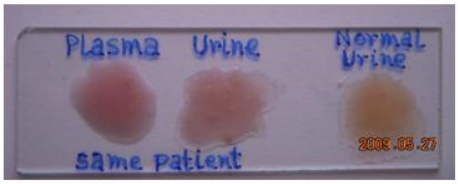


Fig: 6-b

Fig: 6-c Dried slide

(Table/Fig 6)



(Table/Fig 7)

Backdrops Of This Reagent

This is not stable in a humid atmosphere and develops a light green colour. Once the light green colour develops, the reagent becomes nonfunctional.

Conclusion

A solid phase single reagent for the detection of ketone bodies has been successfully developed and other laboratories can prepare the reagent on their own using this formulation.

References

[1] Powers A.C. Diabetes Mellitus in Harrison's Principle of Internal Medicine, 17th ed. p: 2152-79, 2005

[2] Caraway W T and Watts NB. Carbohydrates in Tietz N. W. et al Textbook of Clinical Chemistry, 2nd ed. p: 808-10, 1986