

## METHOD OF MEASURING THE TOTAL KETONE BODY AND A SAMPLE REAGENT

### FIELD OF INVENTION

The present invention relates to a method of assaying the total ketone body in a sample, and more specifically, relates to a method of measuring the total amount of acetoacetic acid and 3-hydroxybutyric acid in body fluid, particularly in blood serum, blood plasma or urine (hereinafter referred to as the total ketone body), which is known as a diagnosis marker of diabetes in the field of clinical tests, and test reagents for the assay. The present invention provides a simple and highly precise assay method using an enzyme, and test reagents for carrying out the assay.

### PRIOR ART

When saccharometabolism is due to lack of supply of carbohydrate or an impediment in its utilization, stress, an excess of exercise on diabetes, fat metabolism accelerates substitutionally, and a large amount of a fatty acid is released from adipose tissue. It is known that the released fatty acid is beta-oxidized at a hepatic mitochondria and that as a result the amount of acetoacetic acid and 3-hydroxybutyric acid in body fluid increases. It is useful particularly for diagnosing diabetes to assay these compounds, and is deemed to be extremely useful clinically (Yukio Shigeta: *Keton Body*, *Nihon Rinsho*, 40, autumn special issue, 250 (1982)).

As a method of assaying a ketone body, the following have been reported:

- (1) according to gas chromatography (refer to *Analgt. Biochem.*, 47, 235 (1972); hereinafter referred to as GC);
- (2) a semi-quantitative method utilizing the coloration of acetone and acetoacetic acid by nitroprusside (hereinafter referred to as a semi-quantitative method);
- (3) a method of subjecting acetoacetic acid to a color reaction by coupling it with diazonium salt of p-nitrophenyldiazonium fluoroborate and subjecting it to colorimetry (refer to Japanese Patent Public Disclosure No. 15004/1980); or a modification thereof, a method comprising reacting acetoacetic acid with p-nitrophenyldiazonium fluoroborate in the presence of a surface active agent, alkalizing it to form a stable azo compound and subjecting it to colorimetry (refer to Japanese Patent Public Disclosure No. 5959/1984; hereinafter referred to colorimetry);
- (4) an enzyme method using 3-hydroxybutyric acid dehydrogenated enzyme (refer to Yukio Shigeta: *Nihon Rinsho*, 38, 638 (1980), and Williamson D. H.: *Biochem. J.* 82, 90-96 (1962); hereinafter referred to as an enzyme method); and
- (5) a method utilizing an enzyme cycling reaction according to 3-hydroxybutyric acid dehydrogenated enzyme (refer to Japanese Patent Public Disclosure No. 158779/1992; hereinafter referred to an enzyme cycling method).

Samples for the measurement of a ketone body are human body fluid, particularly blood serum or blood plasma of diabetic patients. A ketone body is a general term for acetoacetic acid and 3-hydroxybutyric acid; generally 3-hydroxybutyric acid occupies a higher concentration than acetoacetic acid and the difference tends to become large at sick conditions. Since acetone easily gasifies, is unstable and

exhausted through expiration, it is not often assayed generally.

Hence, the ketone body according to the present invention means "a combination of acetoacetic acid and 3-hydroxybutyric acid".

Problems of conventional methods of assaying the ketone body are as follows.

The GC method is a method comprising chemically converting acetoacetic acid and 3-hydroxybutyric acid to acetone and measuring the acetone. Pretreatment of a sample is essential in a method which gives rise to problems in treating a large number of samples in a short time.

The semi-quantitative method is useful for providing an index for urgent treatment such as an intake of carbohydrate and the administration of insulin. However, since the method functions on the basis of detection of the active hydrogen of a molecule, it is disturbed by a compound having active hydrogen present in a sample, and further it has an essential defect that 3-hydroxybutyric acid having no active hydrogen cannot be detected. In addition, it has such a low sensitivity that only acetoacetic acid of more than 0.5 mM and acetone of more than 2 mM can be detected, and hence it is impossible around normal values to determine a disease state.

Though colorimetry is excellent in terms of its high sensitivity and capability of carrying out an assay in a short time, it is necessary in advance of a measuring step to subject a sample to a deproteinizing treatment or dialysis. Hence, it is not suitable for application to an automatic analyzer for measurement of many samples at one time.

An enzyme method gives rise to no special problem for the measurement of 3-hydroxybutyric acid. In the case of measuring acetoacetic acid, however, it has a defect that since the consumption of reduced-type nicotinamide adenine dinucleotide (reduced-type NAD) is measured, a slight absorbance decrease is measured in the high absorbance range, and hence that measurement precision is sacrificed.

An enzyme cycling method has advantages such that its sensitivity is extremely high and that no pretreatment of a specimen is needed, and that it can be easily applied to an automatic analyzer for measuring many samples at one time. However, since the assay employs thionicotinamide adenine dinucleotide (thio NAD) which is difficult to function as a coenzyme of 3-hydroxybutyrate dehydrogenase, an unusually high concentration of 3-hydroxybutyrate dehydrogenase is required. Hence, this method is uneconomical. In addition, since the maximum absorption wavelength of the reduced-type thio NAD, an analogue of NAD, is around 400 nm and measurement is performed at that wavelength, it is prone to be affected by hemolysis and the presence of bilirubin.

### SUMMARY OF THE INVENTION

The object of the present invention is to provide a convenient and rapid method of assaying a ketone body in a sample. The method can be performed with high precision, and requires no pretreatment of the specimen. Thus it solves the defects of conventional methods of measuring a ketone body as described above, and moreover provides a method of measurement by means of a general-purpose automatic analyzing device and a reagent therefor.

The present invention relates to a method of assaying the total ketone body in a sample, which comprises the steps of:

- (1) converting acetoacetic acid in the sample to 3-hydroxybutyric acid;