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THE STABILITY AND AUTOMATIC DETERMINATION OF KETONE BODIES IN BLOOD SAMPLES TAKEN IN FIELD CONDITIONS

By

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TYÖPPÖNEN, J. & K. KAUPPINEN: *The stability and automatic determination of ketone bodies in blood samples taken in field conditions.* Acta vet. scand. 1980, *21*, 55—61. — An automated, spectrophotometric determination of blood acetoacetate and β-hydroxybutyrate was developed with a Gilford 3500 autoanalyzer. The stability of ketone bodies was studied in different conditions. An immediate precipitation with 0.6 M perchloric acid and cooling the sample effectively prevent the loss of acetoacetate from samples during transport to the laboratory (at 4°C a 6 % loss of acetoacetate was noted during 24 h). Freezing the sample makes it practically stable (less than 2 % loss of acetoacetate per week during a study lasting 2 months). At room temperature (20°C) the sample's acetoacetate was instable and disappeared with a rate of 6 % per h. β-hydroxybutyrate was stable in precipitated samples. Because the precipitation also retains the sample's glucose, 3 main parameters for the indication of ketosis could be analyzed automatically from the same sample with a total capacity of 40 samples in 2½ h.

acetoacetate; bovine ketosis; Gilford 3500 analyzer; glucose; β-hydroxybutyrate.

Bovine ketosis is one of the main problems in Finnish dairy cattle husbandry. It causes considerable economical losses. Objective assay methods are needed, if the incidence of bovine ketosis is to be studied. On the other hand primary ketosis is very difficult to induce experimentally and therefore it is also necessary to make wide field studies when approaching this problem.

Bovine ketosis means an increase of ketone bodies in the body fluids, and a decrease in the plasma level of glucose. The main ketone bodies in blood and those usually used for analysis are acetoacetic acid and β-hydroxybutyric acid. Modern laboratory

technology makes it possible to analyze a large amount of blood samples. A common problem in field studies, however, is the chemical instability of acetoacetate in blood samples (*Williamson et al.* 1962, *Mellanby & Williamson* 1974). Because the aim of this study was to analyze all 3 main parameters for ketosis in blood samples taken in field conditions, a thorough study was made of the sample stability at different temperatures with different treatments. Because our analyzer (Gilford 3500 system) had no program for analyzing the ketone bodies, a program of high capacity was developed for measuring acetoacetate, β-hydroxybutyrate and glucose from the same sample.

MATERIALS AND METHODS

The present method for determination of acetoacetate (AA) and β-hydroxybutyrate (BHB) is enzymatic, kinetic and based on reversible reaction catalyzed by β-hydroxybutyrate dehydrogenase (BHBDH) (*Williamson et al.* 1962):

$$AA + NADH + H^+ \leftrightarrows BHB + NAD^+$$

The principle for measuring is the reversible redox reaction of the coenzyme NADH/NAD which causes a change in absorbance. A fall in absorbance at 340 nm is an expression of AA concentration (pH 6.9), whereas a rise in absorbance is an expression of BHB concentration (pH 9.5) (*Wildenhoff* 1970). The present method is developed mainly by combining the methods of *Price et al.* (1977) and *Hansen & Freier* (1978) with necessary modifications for the Gilford 3500 analyzer. The determination program is the same for both acids: General kinetics substrate at a temperature of 37°C, wavelength 340 nm, preincubation time 5 s, incubation time 15 s, measuring time 30 s, sample volume 0.1 ml and reagent volume 1.0 ml. Concentrations in reaction mixture: AA-assay: 0.37 mM NADH and 75 u/l BHBDH in 0.3 M pH 6.9 Na-phosphate buffer. BHB-assay: 0.37 mM NAD^+ and 75 u/l BHBDH in 0.1 M pH 9.5 tris buffer. Standards: Li-acetoacetate (Sigma Chemical Co., Ltd.) and Na-DL-3-hydroxybutyrate (Boehringer Corp., Ltd.). The enzyme and the coenzymes were obtained from Boehringer Corp., Ltd. For the determination of glucose in samples a Gilford standard method was used: GOD-PAP (Trinder method) with Boehringer reagents. To compensate the dilution of the blood sample in precipitation, the sample volume was increased from 10 to 50 µl in glucose determination.

The stability of AA (0.5 mM) was first studied in (a) heparinized whole blood, (b) in a blood sample precipitated with 0.6 M perchloric acid (PCA) and (c) in neutralized blood-PCA-extract. The experiment was carried out at 4°C and at —20°C during 4 days. Secondly, the stability of AA (0.5, 1.0 and 2.0 mM) and BHB (2.5, 5.0 and 10.0 mM) was examined in acidic PCA-samples stored at 20°, 4° and —20°C during varying periods up to 8 weeks. In field studies the blood samples were obtained randomly from 142 cows in 21 herds. For ketotic values blood was sampled from 19 cows that showed clear clinical symptoms for ketosis (Ketostix Ames positive in milk and urine). The blood samples were drawn from the mammary vein, 0.5 ml heparinized blood was immediately pipetted to 2.0 ml cold 0.6 M PCA, and the tube was shaken thoroughly. The precipitated sample was kept at 4°C and frozen during the next 6 h at —20°C. In the laboratory the sample was centrifuged at 3000×g for 5 min, and the supernatant was neutralized with 3 M-KOH and placed on ice. After 30 min the $KClO_4$-precipitate was removed by centrifugation as before. The clear supernatant was used for analysis of AA, BHB and glucose.

RESULTS AND DISCUSSION

In whole blood acetoacetate was instable, as expected (*Mellanby & Williamson* 1974). After 4 days at 4°C the recovery of added acetoacetate in whole blood samples was only 50 %. At —20°C the recovery was 67 %. Therefore the transport of whole blood samples is not possible without stabilization. For that purpose the immediate precipitation of the proteins with 0.6 M PCA was chosen. As in an acidic sample there is a risk for loss of acetoacetate via decarboxylation to acetone, the stability of the acidic sample was compared with a neutralized sample at 2 different temperatures. At 4°C the AA recovery in the acidic sample was below 80 % after 4 days, but in the neutralized sample about 100 % in the same time. At —20°C the differences in recoveries disappeared; after 4 days the recoveries in both the acidic and neutralized sample were near 100 %. As in field conditions it is easier to freeze the samples than neutralize them, the simplest way for ensuring their stability in these conditions is the immediate precipitation with freezing.

In the second phase of the stability studies it was examined, how different concentrations of both AA and BHB in the same

sample behave at different temperatures as a function of storing time, when stored acidic after PCA precipitation. At room temperature (20°C) the recovery of added AA in samples after 24 h was only 40 %, but the concentration of added BHB in the same sample was unchanged. Thus the loss of AA is about 2.5 % per h in the acidic sample at room temperature. *Price et al.* (1977) made the same observation. By refrigeration (4°C) for 5 days the loss of AA in samples was a mean of 6 % per day. This finding is in agreement with the studies of *Williamson et al.* (1962) and *Price et al.* The BHB recoveries in the same samples (4°C, 5 days) were around 100 %.

Both ketone bodies were very stable at —20°C. Stored in this way the disappearance of both AA and BHB in acidic samples during a study for 2 months was a mean below 2 % per week. In all cases, the stability of the ketone bodies was independent of the added concentrations.

One of the purposes in developing the present method was the high analyzing capacity, necessary e.g. in screening the ketosis status and frequency in a large scale. In this work the 3 important parameters for indication of ketosis, AA, BHB and glucose, could be analyzed automatically in the same sample with a total capacity of 40 samples in 2½ h.

It is very difficult to distinguish between normal, subclinical and ketotic values when blood concentrations of ketone bodies

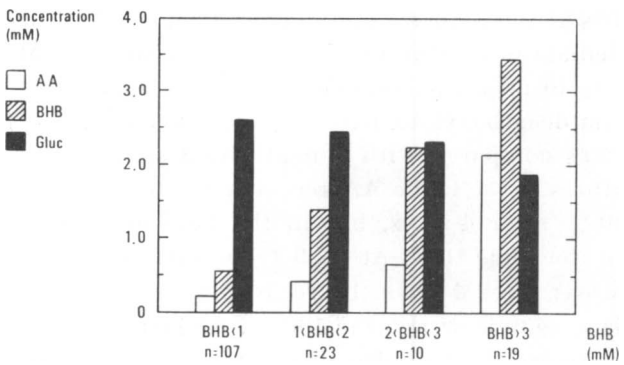

Figure 1. The interrelationship of blood ketone bodies and glucose in different degrees of ketosis. The grouping is theoretical and is based on β-hydroxybutyrate values at 1 mM intervals. The columns represent means in each group. AA = acetoacetate, BHB = β-hydroxybutyrate, Gluc = glucose. For further explanation see text.

are moderately elevated. *Baird et al.* (1968) measured AA concentrations at 0.01—0.02 mM for normal cows and at 0.5—1.1 mM for ketotic cows. *Halse et al.* (1976) reported basal concentrations of AA below 0.06 mM and mean AA values for ketotic cows of 1.8 mM. *Møller & Halse* (1978) used the AA concentration 0.85 mM to distinguish between moderate and heavy ketosis. *Baird et al.* (1968) considered blood BHB concentrations of 0.2—0.4 mM to be normal and those between 3 and 5 mM ketotic. *Kelly* (1977) distinguished between low BHB values (0.3—1.0 mM) and high BHB values (1—4 mM). Blood glucose concentration is usually used as an additional indicator for metabolic conditions. With increasing concentrations of ketone bodies there is a decreasing concentration of blood glucose, the lowest values being noted in lactating cows during starvation (1.73 mM; *Baird et al.* 1972).

For making reference values to the present method blood was sampled randomly from 142 cows in 21 herds. For ketotic values blood was sampled from 19 ketotic cows, as described in section "Materials and methods". The immediate precipitation of proteins prevents the glycolytic action in erythrocytes, and the glucose concentration in samples remains unchanged.

To get a picture of the distribution of the analyzed material, the 161 values were grouped on a basis of the β-hydroxybutyrate concentration in samples at 1 mM intervals (Fig. 1). The rather linear increase of BHB is followed by a slower, non-linear increase of AA and a slighter decrease in glucose concentration. The most marked changes in AA and glucose concentrations take place first at the highest levels of BHB. To divide the values in normal, subclinical and ketotic values it seems, with reference to the literature and to clinical signs of the animals, that cows with BHB concentration below 1 mM are normal, those with BHB

Table 1. Concentrations of ketone bodies and glucose in deproteinized blood samples from dairy cows (mean ± s) measured with Gilford 3500 autoanalyzer. For further explanation see text.

	n	AA (mM)	BHB (mM)	Glucose (mM)
Normal	109	0.22 ± 0.07	0.56 ± 0.22	2.62 ± 0.67
Subclinical	33	0.48 ± 0.21	1.66 ± 0.49	2.42 ± 0.75
Ketotic	19	2.14 ± 0.89	3.45 ± 1.88	1.89 ± 0.55

between and 1 and 3 mM are subclinical, and when BHB values are above 3 mM the cows are ketotic (Table 1). Such sharp grouping is, of course, theoretical and uncertain especially with respect to subclinical values.

The greatest use for the precise determination of the blood concentrations of ketone bodies and glucose is when each cow is its own control, e.g. in following a medical treatment or in attempts to clarify the causes of primary ketosis.

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SAMMANFATTNING

Stabilitet och automatisk bestämning av ketonkroppar i blodprov tagna under fältförhållanden.

Man har utvecklat en automatisk spektrofotometrisk bestämningsmetod av acetoacetat och β-hydroxybutyrat i blod med Gilford 3500 autoanalyzer. Ketonkropparnas stabilitet har undersökts under olika förhållanden. Omedelbar precipitering med 0,6 M perklorsyra och nedkylning av provet förhindrar effektivt förlust av acetoacetat under transporten till laboratoriet (vid 4°C noterades en förlust av 6 % i acetoacetatkoncentrationen under ett dygn). Nedfrysningen av provet gör att det blir praktiskt taget stabilt (mindre än 2 % förlust av acetoacetat per vecka under en två månaders undersökning). Vid rumstemperatur (20°C) var acetoacetatet instabilt och koncentrationen minskade med 6 % i timmen.

β-hydroxybutyratet var stabilt i de precipiterade proven. Emedan precipiteringen också kvarhåller provets glukos, kan man ur samma prov automatiskt analysera tre huvudparametrar för indikering av ketos med en total kapacitet av 40 prov på 2,5 timmar.

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