Fluorometric Determination of β -Hydroxybutyrate in Milk and Blood Plasma

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ABSTRACT

Determination of β -hydroxybutyrate (BHBA) in blood and milk samples is an important tool in the diagnosis of ketosis in dairy cattle. Apart from semiquantitative cow-side tests, well-established laboratory methods exist for measurements in blood serum or plasma. These spectrophotometric methods are, however, neither convenient nor reliable when transferred to analyses of milk. Due to its nontransparent nature, milk needs extensive pretreatment if traditional analyses are to be used. This paper describes a fluorometric determination of BHBA that is useful without pretreatment in opaque matrices such as milk and in blood plasma. The method is easy to automate, saves labor expenses, and is inexpensive. The analytical accuracy and precision are reliable for intensive as well as large-scale analysis; for example, in-line sampling from automatic milking systems. Analysis of 2500 random milk samples showed a BHBA content ranging from 10 to 631 μM (mean 49 μM). Furthermore, selected samples ($n = 295$) from diagnosed ketotic animals taken on d -35 to $+35$ from peak level ranged from 10 to 684 μM (median 79 μM , mean 141 μM). Using the same 1240 blood plasma samples, the fluorometric method was closely correlated with a traditional spectrophotometric method ($r = 0.987$). Hemolysis of samples does not appear to affect the fluorometric determination of BHBA.

(**Key words:** β -hydroxybutyrate, ketosis, milk, blood)

Abbreviation key: H_1 and H_2 = high internal BHBA controls, L_1 and L_2 = low internal BHBA controls.

INTRODUCTION

The ketone bodies in the intermediary metabolism (acetone, acetoacetate, and BHBA) are relatively simple chemical structures. β -Hydroxybutyrate is synthesized from absorbed butyrate in the rumen epithelium of ru-

minants and by the ketogenesis of the liver cells in the conversion of long-chain fatty acids from fat mobilization. Acetoacetate and BHBA are freely distributed and transported in the blood, and seem interconvertible in various tissues; in fact, BHBA seems to contribute to the overall energy consumption in the body to a non-negligible degree (Bruss, 1997).

Acetoacetate and BHBA are moderately acidic compounds and consequently influence the acid-base balance in the organism (ketoacidosis). However, several forms of ketosis have been suggested in the modern cow, distinguishable from each other by the origin of the syndrome [the reader is referred to Bruss (1997) for further details]. Irrespective of the origin of the ketosis, the level of ketone bodies in the organism may be too high, indicating a situation of inappropriate metabolism that should be corrected for the sake of the animal's health.

Analyses of breath, urine, milk, and blood have traditionally been included in the surveillance of the health status of the cow. Cow-side methods using strips, tablets, and powders have been useful for the semiquantitative determination of ketones. In the laboratory, the enzymatic conversion of acetoacetate to BHBA (with the concomitant development of NAD^+ from NADH) may be assessed as a decrease in absorbance spectrophotometry at 340 nm. The reverse process, the oxidation of BHBA to acetoacetate (production of NADH), may accordingly be assayed as an increase in absorbance. The change in NADH/NAD^+ concentrations has also been detected directly by fluorometry (Greengard, 1956; Young and Reynold, 1966). The traditional enzymatic and spectrophotometric determinations are generally considered reliable and precise. Detailed procedures are given by Williamson et al. (1962), Mellanby and Williamson (1974), and Williamson and Mellanby (1974) and in more automated editions by Ozand et al. (1975), Työppönen and Kauppinen (1980), and Harano et al. (1985).

One obvious advantage of using milk instead of blood for analysis of ketosis is the noninvasive nature of milk sampling. However, the spectrophotometric assay for determination of BHBA in milk has not been directly suitable, because of the opacity of milk. Opacity of the

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sample is incompatible with spectrophotometry. Consequently, pretreatment of milk samples has been necessary to obtain a transparent sample before photometric determination. Mäder (1980) used precipitation with perchloric acid and subsequent neutralization; Andersson and Lundström (1984) precipitated the milk protein with rennet at 40°C for 12 h and centrifuged before assaying; Dirksen and Breitner (1993) used repeated centrifugation and precipitation with uranyl acetate; and Nielsen et al. (2003) used precipitation, centrifugation, and special harvesting of the infranatant to overcome this problem.

The present paper describes a fluorometric method suitable for assessment of BHBA in any fluid irrespective of opacity, including milk without pretreatment.

MATERIALS AND METHODS

Animals and Sampling

Milk and blood samples were harvested from an experimental dairy farm with 55 Danish Holstein, 55 Red Danish, and 40 Danish Jersey cows in a loose housing system. All cows were fed a TMR ad libitum consisting of grass silage, corn silage, rapeseed cake, barley, sugar beet cobs, minerals, and vitamins. Seventy of these cows, representing all 3 breeds and parities from 1 to 4, were blood sampled twice weekly for the first 9 wk after calving. Blood samples ($n = 1240$) were collected by venipuncture of the coccygeal vein/artery using 10-mL heparinized tubes, which were put on ice immediately after collection. Plasma was subsequently harvested by centrifugation at $2000 \times g$ for 20 min at 4°C and stored at -18°C until chemical analysis.

Harvested plasma was subjectively scored for hemolysis on a 4-point scale (0 to 3; 0 = no hemolysis, 3 = severe hemolysis). All 150 cows were milked with an automatic milking system (3 boxes, 2.4 milkings/cow per d) and composite (metered) milk samples were collected automatically from every milking, as part of the daily routine at the experimental farm. Milk tubes were pretreated with Bronopol (2-bromo-2-nitro-1,3 propanediol) solution to obtain 200 mg/L in the final volume. The automatic milk samplers were emptied in the morning and the afternoon; milk samples were kept at 4°C until analysis. Milk samples were analyzed in the laboratory within 24 h of sampling without freezing. Two thousand five hundred milk samples from random days in June 2004 were measured. These 2500 samples represented all 3 breeds and parities from 1 to 4. Additionally, 295 samples were analyzed representing 7 cows that were diagnosed and treated for ketosis. The samples were selected on d -35 to +35 from peak BHBA.

Chemical Analyses of Milk

The milk BHBA analyses were based on the enzymatic method introduced by Williamson et al. (1962), (step a); however, a second process was coupled to the oxidation of BHBA, i.e., a subsequent oxidation of the developed NADH with rezasurin thereby producing equimolar levels of the fluorescent compound resorufin (Guilbault et al., 1969), (step b):

D- β -OH-butyrate dehydrogenase

NAD oxidoreductase, EC 1.1.1.30

The process is reversible, but is displaced to the right under slightly alkaline conditions.

The NADH + H⁺ developed in this process is coupled to a chromophore, and a fluorescent product develops:

diaphorase,

lipoyl dehydrogenase,

NAD oxidoreductase, EC 1.8.1.4

The developed resorufin is detected fluorometrically as emission at 575 nm after excitation at 544 nm.

A side reaction, i.e., a process that may produce NADH + H⁺ under the same conditions, namely the oxidation of lactate to pyruvate by L-lactate dehydrogenase (EC 1.1.1.27), was prevented by adding oxamic acid to the substrate (Novoa et al., 1959; Harano et al., 1985).

Standards and controls were manufactured from a batch of milk originating from a single cow in late lactation. The milk was long-time pasteurized (75°C, 30 min), supplied with Bronopol to 200 mg/L, and DL-BHBA (Sigma H-6501; Sigma Chemical Co., St. Louis, MO) to obtain 0, 60, 120, 200, 360, 560, and 800 μM D-BHBA (standards), and 80 and 400 μM D-BHBA (internal controls).

Milk samples were pipetted, diluted, and distributed for different analyses using a Biomek 2000 workstation (Laboratory Automation Workstation, Beckman Coulter, Fullerton, CA). Reagents for BHBA assays were added in the robotic system as well as in the fluorimeter, Fluostar (BMG Labtechnologies, 77656 Offenburg, Germany). Analyses were performed in 96-well plates; 2×7 standards and 2×2 control samples were used for every analysis and plate. The fluorescence readings were adjusted within the interval blank (0 μM) and 800 μM BHBA. The obtained standard curve

is a straight-line regression (the regression coefficient was never below 0.99).

The analytical conditions were: Tris-buffer pH 8.5: 0.06 M; oxamic acid: 7.1 mM; NAD: 3.4 mM; rezasurin: 1.4 mM; Triton X-100: 0.13% (vol/vol); D-BHBA dehydrogenase: 0.21 U/mL (Roche 127 841, Copenhagen, Denmark); diaphorase: 1.8 U/mL (Toyobo Enzymes, DAD 301, Osaka, Japan). The volume fraction of the sample was 0.14. The procedure was designed as an end-point assay, where the fluorescence was read 18 min after sample addition.

Quality control of the analyses was conducted on 20 plates assayed on consecutive days. The accuracies of the 80 μ M low internal controls (L_1 and L_2 , respectively) were 87.5 ($\pm$ 12.2 SD) and 91.1 μ M ($\pm$ 11.1 SD). Within-plate precision was 5.6% (n = 38). Between-plate precision (CV) was 14.0 and 12.1% for L_1 and L_2 , respectively. Corresponding accuracy values for the high internal controls (400 μ M; H_1 and H_2) were 383.7 ($\pm$ 32.1 SD) and 396.8 μ M ($\pm$ 26.4 SD), respectively. Within-plate precision (CV) was 3.0% (n = 38). Between-plate precision was 8.4 and 6.7% for H_1 and H_2 , respectively. The detection limit of the analyses was assessed using 38 plates each with duplicate determinations of basis milk (0 standards). The mean standard deviation (SD) between standards was 11.5 μ M; the conventionally used detection limit ($3 \times$ SD) is approximately 35 μ M.

Chemical Analyses of Plasma

Fluorometric detection. The fluorometric determination of plasma BHBA was based on the procedure for milk analysis. However, the concentrations of reagents and samples were changed, and standards and controls were manufactured with a different matrix.

The analytical conditions were: Tris-buffer pH 8.5: 0.09 M; oxamic acid: 8.3 mM; NAD: 4.0 mM; rezasurin: 1.6 mM; Triton X-100: 0.15% (vol/vol); D-BHBA dehydrogenase: 0.25 U/mL; diaphorase: 0.33 U/mL. The fractional volume of the sample was 0.056. The determination was assessed as an end-point assay, where the fluorescence was read 18 min after sample addition.

Standards (in duplicate) representing 0, 0.3, 0.6, 0.9, 1.2, 1.8, and 3.0 mM D-BHBA were prepared based on Tris-buffer (pH 8.5). Controls representing 0.5 (L_1 and L_2) and 1.5 (H_1 and H_2) mM D-BHBA were also prepared and used. Two plasma samples were each analyzed in triplicate for every microplate used.

Quality control of the analyses was assessed on the 17 microplates involved in the analyses. The accuracies of the 0.5 mM controls (L_1 and L_2) were 0.505 mM ($\pm$ 0.050 SD) and 0.509 mM (0.070 SD), respectively. Within-plate precision averaged 4.8%; between-plate precision (CV) was 9.9% for L_1 and 13.7% for L_2 .

The accuracies of the 1.5 mM controls H_1 and H_2 were 1.445 ($\pm$ 0.065 SD) and 1.473 mM ($\pm$ 0.053 SD), respectively. Within-plate precision averaged 2.3%; between-plate precision was 4.5% for H_1 and 3.6% for H_2 .

Plasma controls gave intra-assay precisions (CV) of 4.7 and 3.2% for low and medium level contents (0.72 and 1.47 mM), respectively. The corresponding interassay precision was 8.7 (low) and 9.1% CV (medium level).

Spectrophotometric detection. Blood plasma was analyzed for BHBA using an auto analyzer (ADVIA 1650, Bayer Corp., Tarrytown, NY). The enzymatic procedure was based on the set-up described by Harano et al. (1985), but with modifications. The reaction conditions in the mixture were: Tris-buffer, pH 8.5: 0.10 M; NAD: 1.34 mM; oxamic acid: 12.8 mM; D-BHBA dehydrogenase: 0.7 U/mL. Plasma made up 5.6% of the reaction mixture. The end-point process was read after 8 min.

Standards were prepared from DL-BHBA (Sigma H-6501) at 0.2, 0.5, 1.0, 2.0, and 3.0 mM D-BHBA. Controls were manufactured independently to contain 0.63 and 1.67 mM D-BHBA.

Quality control of the analyses was conducted using 12 cow plasma samples (range 0.4 to 3.3 mM) and the 2 prepared control samples. Each plasma sample was run 4 times/d, and each control (L and H) was run 3 times/d for 8 d. The intra-day sample CV was between 0.34 and 0.93% (mean = 0.6%). The inter-day sample CV was between 1.2 and 3.8% (mean = 2.1%). The accuracies of controls were 0.63 mM ($\pm$ 0.0068 SD) and 1.66 mM ($\pm$ 0.0068 SD), resulting in accuracy biases of low and high controls being -0.6 and -0.8%, respectively.

Plasma samples scored from 0 to 3 on the hemolytic scale were analyzed with and without sample blanking procedure.

RESULTS

Milk samples were analyzed for BHBA using the coupled reaction process and fluorometric detection. The BHBA level in the samples ranged from 10 to 631 μ M; on average, the milk contained 49 μ M. The 0.05, 0.50, and 0.95 quantiles were 18, 42, and 88 μ M, respectively. The detailed frequency distribution is shown in Figure 1.

Furthermore, samples from 7 selected cows diagnosed to be ketotic (winter/spring 2004), were analyzed approximately 35 d before the BHBA level peaked until 35 d after (Figure 2). This material represented 295 samples; mean value was 141 μ M and the median represents 79 μ M BHBA.

Blood plasma samples were analyzed according to the traditional enzymatic spectrophotometric method.

Figure 1. Frequency distribution of 2500 milk samples, analyzed for BHBA by the fluorometric method. Samples were taken in June 2004 among 150 cows irrespective of their present history of ketosis. The measuring range was 10 to 631 μM , mean content was 49 μM BHBA, and the 0.05, 0.50, and 0.95 quantiles were 18, 42, and 88 μM , respectively.

The range of BHBA values was from 0.35 to 12.0 mM ; the average value was 1.13 mM ; and the 0.05, 0.50, and 0.95 quantiles were 0.55, 0.90, and 2.69 mM , respectively.

The same blood plasma samples were analyzed for BHBA using the fluorometric method: Minimum and maximum contents of the 1240 samples were 0.28 and 12.8 mM , respectively. The mean value obtained by the

Figure 2. Milk from 7 cows with a recognized ketosis status was analyzed by the fluorometric method during approximately 70 d, $n = 295$. Mean BHBA was 141 μM . Focusing on d -6 to +6 around the peak BHBA level ($n = 90$), the average BHBA content was 233 μM , and the 0.10 and 0.90 quantiles were 36 and 435 μM , respectively.

Table 1. Hemolyzed plasma samples were measured for BHBA using a spectrophotometric method, with and without sample blank, and by the fluorometric method. Figures in parentheses are in proportion to the fluorometric determination.

Hemolytic score	Samples, no.	Spectrophotometric method		Fluorometric method
		Mean, mM Without sample blank	Mean, mM With sample blank	Mean, mM
1	31	0.98 (1.56)	0.68 (1.08)	0.63
2	14	1.30 (1.55)	0.77 (0.92)	0.84
3	3	1.95 (2.60)	0.70 (0.93)	0.75

analyses was 1.10 mM, and the 0.05, 0.50, and 0.95 quantiles were 0.45, 0.82, and 2.81 mM, respectively.

Forty-eight plasma samples were scored from 0 to 3. These samples were analyzed spectrophotometrically with and without blanking and by the fluorometric method. Details of these analyses are shown in Table 1. The blanked, spectrophotometric BHBA analysis was more closely correlated with the fluorometric determination than the nonblanked analysis ($r = 0.929$ vs. $r = 0.697$; $n = 48$).

A comparison between the 2 analytical determinations, i.e., the spectrophotometric and the fluorometric, revealed a very close association ($r = 0.987$, Figure 3).

DISCUSSION

We obtained extremely high precision (low CV%) in both intra- and interassay determinations of our spec-

trophotometric BHBA method of blood plasma. The precision is apparently inferior when using the fluorometric method. However, when comparing blood plasma and milk using the fluorometric measurement, it seems that the milk matrix appears less homogeneous and therefore may cause a higher coefficient of variation of the precision.

The present fluorometric determination of BHBA appears to be very reliable compared with the well-established spectrophotometric method, which is practiced in matrices such as urine, blood plasma, other biological fluids, and precipitated milk. The obvious advantage of using the fluorometric method is that milk or other opaque liquids do not need pretreatment to obtain a transparent status; the developed fluorophore is easily read in nontransparent liquids.

Avoiding pretreatment is crucial if large quantities of milk have to be analyzed. Addition of exact volumes

Figure 3. Comparison between 2 analytical methods in the determination of BHBA in blood plasma. Both methods are based on enzymatic conversion of BHBA and NAD to acetoacetate and NADH. In the fluorometric method, NADH is coupled to a second substrate in the development of a fluorescent product.

of acids or other precipitating components, and subsequent centrifugation and pipetting steps to obtain a clear supernatant are laborious. Furthermore, the precipitating step itself may be a source of contamination, disturbing the analyses for BHBA; either the precipitation is a one-step procedure or is followed by a concomitant neutralization and if necessary, further centrifugation.

Analyses of BHBA in blood plasma using the traditional enzymatic spectrophotometric method are susceptible to background absorbance in the sample, especially if blood samples are hemolyzed (Jacobs et al., 1992; Duffield et al., 1998). Insertion of a proper sample blank or use of efficient second wavelength detection in the analyses may minimize this problem. Hemolyzed samples, not corrected for background absorption, will appear to have a higher BHBA concentration than if a correction is used. The present fluorometric method appears not to be susceptible to hemolysis. Hemolyzed blood plasma samples analyzed by the fluorometric method were only narrowly correlated with the spectrophotometric determination if plasma blank (background) was taken into account. The latter demonstration makes it plausible that blood in milk samples due to mastitis will not interfere with the BHBA assessment when using the presented method.

In conclusion, the presented fluorometric determination of BHBA is a valid method for analysis of plasma or whole milk without pretreatment. The assay is easily automated to permit the handling of large numbers of samples. The method is ideal for large-scale in-line sampling of milk.

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