

RELATIONSHIP BETWEEN CONCENTRATION OF CITRATE AND KETONE BODIES IN COW'S MILK

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The authors' hypothesis is that the members of the tricarboxylic acid cycle (TCA cycle) such as citrate decrease in association with increased ketone body formation. To prove this hypothesis the connection between ketone bodies and citrate formation of milk was studied. A fluorimetric method was used to determine citrate and a headspace sampling gas chromatographic (GC) method was developed for determination of ketone bodies. Under real conditions of milk sampling, transport and storage, preserved milk samples of 119 clinically healthy dairy cows obtained in the 48 hours after milking were investigated. A low level of acetoacetate (ACAC) was found in all samples. This fact can be explained by the spontaneous decarboxylation of acetoacetate during sample storage (previously decarboxylated acetoacetate = pdACAC) and, consequently, the majority of the amount of acetoacetate in the samples (AC+pdACAC) appeared in the measured acetone concentrations. Based on the measured acetone concentration of milk samples two groups were formed retrospectively: HA (high-acetone) group (n = 41) with an AC+pdACAC concentration of > 0.4 mmol/l and a LA (low-acetone) group (n = 78) with an AC+pdACAC level of ≤ 0.4 mmol/l. In the milk of cows of Group HA a positive correlation (r = +0.623) and linear connection between acetone (AC+pdACAC) and β-hydroxybutyrate (BOHB) levels was found [BOHB = 2.491 + 0.586 × (pdAC + ACAC)]. Furthermore, in this group a negative correlation between citrate and BOHB and AC+pdACAC was also established (r = -0.579). Focusing on the results of this group the authors found a significant drop of AC+pdACAC and citrate during the metabolically critical first 1–4 weeks of lactation. For this reason they suggest that simple, easy, automated methods (i.e. flow injection analysis, Fourier transformation infrared analysis) should be introduced for the simultaneous determination of acetone and citrate concentration in milk to make the evaluation of the energy status of high-producing dairy cows easier and more certain.

Key words: Cow's milk, ketone bodies, citrate

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It is a well-known fact that the content of milk constituents changes in several disorders of energy metabolism. Even in healthy ruminants the inadequate energy supply during late pregnancy and early lactation forces ketogenesis, leading to increasing levels of ketone bodies (acetone, acetoacetate and β -hydroxybutyrate) in body fluids, resulting in ketonaemia, ketonuria and in lactating animals even in ketolactia. This condition is associated with decreased body condition and milk yield, impaired reproductive performance and increased risk of clinical ketosis. The early detection of elevated levels of ketone bodies during the subclinical stage of energy deficiency is highly recommended as on this basis appropriate corrections in the herd management can be introduced to reduce the development of clinical ketosis and consequent losses of profits from milk production (Tóth et al., 1989; Kégl and Gaál, 1992; Fekete et al., 1999; Geishauser et al., 2000).

Nowadays, under field conditions, checking of the β -hydroxybutyrate and/or acetoacetate content of the blood plasma, serum, urine and milk is used to detect energy deficiency and to diagnose ketosis with test strips or other simple, inexpensive chemical reagents such as the Rothera- or Ross-test (Kégl and Gaál, 1992; Geishauser et al., 2000). In spite of the fact that laboratory analysis for ketone bodies is more sensitive than the field tests, biological fluids are rarely sent to the laboratory for ketone body determination alone. However, milk, which is the most easily available biological fluid in dairy cows, is regularly analysed in laboratories for its other constituents (lipids, protein, lactose, urea, cell count, etc.) during lactation. Therefore it is obvious that these regularly investigated milk samples could be analysed for ketone bodies as well using a sensitive standard method. Measurement of milk ketones is a useful tool in the early evaluation of subclinical/clinical ketosis (Fekete et al., 1999; Geishauser et al., 2000).

In addition to elevated ketogenesis, further metabolic changes such as decreased amount of tricarboxylic acid cycle (TCA cycle) intermediates might be expected in the extracellular fluids during energy deficiency. Citrate is an important member of the TCA cycle and has a regulatory effect on acetyl-CoA metabolism in liver mitochondria (Bremer and Davis, 1974). Its amount in cow's body fluids varies with many diseases. The amount of citrate in cow's milk is about 10 mmol/l (Souci et al., 1994). Many papers have reported so far that in healthy cows the citrate content of milk was higher in the early stages of lactation and its concentration gradually declined as lactation advanced (Illek et al., 1997; Khaled et al., 1999). It is interesting that this phenomenon has not been studied in detail so far. Under pathological conditions such as subclinical mastitis, milk citrate decreases proportionally to the degree of inflammation (Oshima and Fuse, 1981).

The effect exerted by the stage of lactation on the presence and distribution of ketone bodies and on concentration of citrate in cow's milk has not been studied. Similarly, no papers are available to clarify whether or not a correlation exists between ketone bodies and citrate in milk.

It is supposed that the concentration of milk citrate changes after calving, especially in the energetically most critical first few weeks of lactation. It is also assumed that a relationship exists between ketone body and citrate levels of the milk. To prove these hypotheses two exact, precise and reproducible but relatively sophisticated, time-consuming and/or expensive methods (headspace gas chromatography and fluorimetry) were used on milk samples of healthy, high-producing dairy cows during the early lactation period.

Materials

Milk samples

Samples under real conditions of sampling, transport and storage were analysed. Milk samples of healthy Holstein-Friesian cows ($n = 119$) were collected randomly at the regular morning milking in 7 dairy herds in Hungary 10–90 days after calving and sent for measuring their composition to the Hungarian Herd Recording Ltd. (Gödöllő, Hungary). An aliquot of all samples arrived at our department on the same day in a well-sealed, cooled ($+4\text{ }^{\circ}\text{C}$) 200-ml bottle. The milk samples were stored at $-18\text{ }^{\circ}\text{C}$ in the laboratory to prevent their further chemical changes, such as spontaneous decarboxylation of acetoacetate to acetone. All chemical analyses were carried out within 48 hours after milking. The origin of the samples and the day of lactation were precisely recorded.

Grouping

Following the laboratory analysis of milk samples two groups were formed retrospectively on the basis of frequency analysis of the measured acetone concentration in the milk. This acetone concentration naturally involves the previously decarboxylised acetoacetate (pdACAC) during sample storage, marked as AC+pdACAC. In the low-acetone group (Group LA, $n = 78$) this milk AC+pdACAC level was $\leq 0.4\text{ mmol/l}$ whereas in the high-acetone group (Group HA, $n = 41$) it was $> 0.4\text{ mmol/l}$ (Fig. 1).

Reagents

Acetone and methyl ethyl ketone reference substances for gas chromatography were purchased from Merck, Germany (catalogue numbers: 100020 and 109709). All other chemicals were of analytical grade and obtained from Reanal Co. Ltd., Hungary.

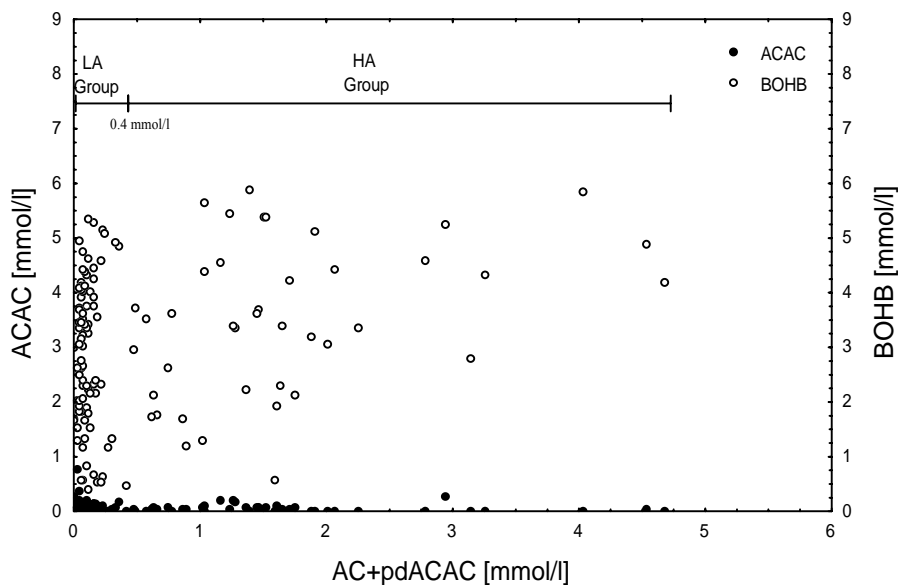


Fig. 1. Distribution of ketone bodies in all milk samples (frequency analysis). LA = low acetone, HA = high acetone, AC = acetone, ACAC = acetoacetate, AC+pdACAC = measured acetone concentration, pdACAC = previously decarboxylised acetoacetate, BOHB = β -hydroxybutyrate

Methods

Gas chromatographic determination of ketone bodies

For determinations of oxidised, reduced and total ketone bodies from milk samples a headspace gas chromatographic (GC) method (Baticz et al., 2001) including appropriate sample preparations was applied. This sample preparation is based on chemical oxidation of the ketone bodies to acetone in three consequent steps as follow:

Step 1: Acetone determination. Ten ml milk and 1 μ l methyl ethyl ketone (internal standard) were pipetted into a 20 ml bottle, capped with rubber septum and adjusted to 60 °C in an air thermostat. Following 5-min incubation with a sampling device having a loop volume of 3 ml the milk samples were injected into the gas chromatograph.

Step 2: Determination of oxidised ketone bodies (acetone + acetoacetate). The same milk samples were treated for further 30 min in a 100 °C thermostat to convert acetoacetate to acetone and were analysed in the same way as in Step 1.

Step 3: Determination of total ketone bodies (acetone + acetoacetate + β -hydroxybutyrate). The same milk samples were treated with 835 μ l oxidising reagent (1.5% potassium dichromate in 7.8 mol/l H₂SO₄) and kept at 100 °C for

further 90 min. After the conversion of β -hydroxybutyrate to acetone the samples were reanalysed with the same procedure as in Steps 1 and 2.

The acetone, acetoacetate and β -hydroxybutyrate concentrations were calculated from the three acetone determinations. The analytical results were obtained by internal standard method.

The GC method was carried out with an adsorption chromatographic system (Carlo Erba, Germany). A 6-ft column packed with Porapak Q 80/100 mesh (Waters Ltd., USA) and equipped with flame ionisation detector was used. Data were collected with HP 35900 ADC interface and were analysed by HP ChemStation software (Hewlett Packard Ltd., USA).

Fluorimetric determination of citrate

A sensitive and specific fluorimetric method for determination of citrate from raw milk samples (Hori et al., 1974) was applied with some modifications.

Before the analysis 4 ml milk was centrifuged in a clear 10-ml tube (10,000 rpm, $g = 3100$) for 20 min to remove the colloidal particles. Distilled water of 0.95 ml and 1 ml metaphosphoric acid solution (2 w/v %) were added to 0.05 ml of milk sample supernatant or standard citrate solution. The mixture was shaken and centrifuged (3000 rpm, $g = 950$) for 10 min. One ml of the supernatant was taken into a digestion tube and 2 ml of *o*-aminothiophenol (*o*-ATH, 0.75 w/v % in 50 w/v % phosphoric acid) solution was added. After replacement of the air with nitrogen gas the test tube was closed and allowed to react at 125 °C for 15 h in an air thermostat. The reaction mixture was cooled with water, then 3 g sodium chloride and 4 ml ethyl acetate were added and the test tube was re-sealed and re-shaken for 5 min. The upper ethyl acetate layer was separated and its fluorescence intensity was measured with excitation at 415 nm and emission at 450 nm by a Jasco FP 920 (Italy) standard fluorimeter.

The citrate content of the milk was calculated from the calibration curve obtained on analyses of 1.0–20.0 mmol/l standard citrate solutions.

Statistical analysis

The results were analysed statistically by frequency analysis, correlation analysis, significance tests and regression analysis using Statistica 5.5 software (StatSoft Inc., USA).

Results

Ketone bodies

Distribution of ketone bodies in raw milk 48 h after sampling was found to be as follows: the acetone (AC+pdACAC) ratios in Groups LA and HA were 3.3 and 81.1% of total ketone bodies, respectively. In both groups very small con-

centration (2–2.5%) of milk acetoacetate (ACAC) was found. Obviously, it was the β -hydroxybutyrate (BOHB) that showed opposite distribution to AC+pdACAC in both groups: in the LA group its ratio was 94.7% while in the HA group it was only 16.3% (Fig. 1).

Relationships among AC+pdACAC, BOHB and citrate are shown in Table 1. The ACAC concentration alone was extremely small in all samples and showed no significant correlation with other parameters, therefore its results are not shown. In the LA group milk pdAC + ACAC did not correlate significantly with BOHB, the reduced ketone body. However, in the HA samples significant positive correlation and linear connection [$\text{BOHB} = 2.491 + 0.586 \times (\text{AC+pdACAC})$] existed between AC+pdACAC and BOHB (Table 1 and Fig. 2).

Table 1

Correlations between ketone bodies and citrate in milk

| | LA Group | | HA Group | |
|-----------|----------------------|---------------------|----------|-----------|
| | Citrate | AC+pdACAC | Citrate | AC+pdACAC |
| BOHB | -0.380* | 0.066 ^{NS} | -0.579** | 0.623** |
| AC+pdACAC | -0.081 ^{NS} | | 0.469** | |

* significant, * $p < 0.01$; ** $p < 0.001$; ^{NS} not significant, $p \geq 0.05$; LA = low acetone, HA = high acetone, AC = acetone, AC+pdACAC = measured acetone concentration, pdACAC = previously decarboxylised acetoacetate, BOHB = β -hydroxybutyrate

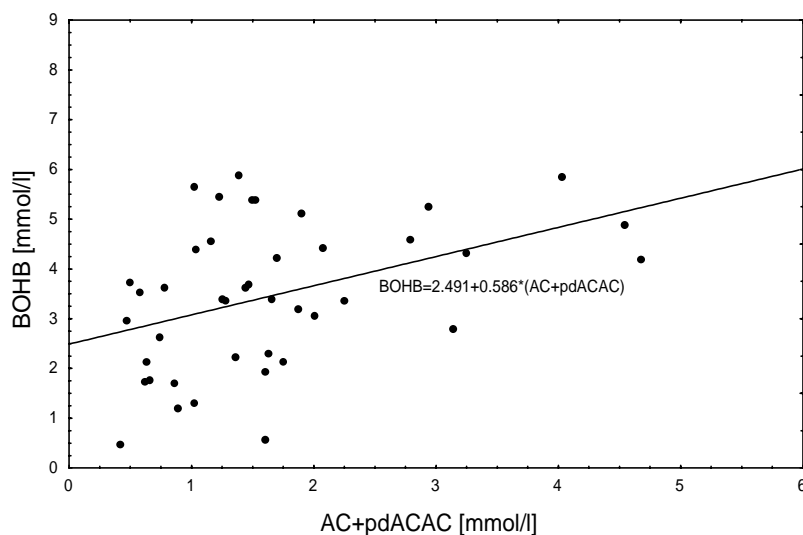


Fig. 2. Relationship between acetone and β -hydroxybutyrate in HA samples. AC = acetone, AC+pdACAC = measured acetone concentration, pdACAC = previously decarboxylised acetoacetate, BOHB = β -hydroxybutyrate

In Fig. 3 AC+pdACAC, BOHB and citrate results are plotted against the days of lactation. The majority of high ketone body levels were measured mainly within the first six weeks of lactation; practically these samples belonged to the HA group. Among ketone bodies the concentration of BOHB slowly elevated till the 60th day of lactation, then its level became approximately constant. Changes in AC+pdACAC concentration showed an opposite tendency, resulting in an about 80–90% drop of AC+pdACAC level by the 60th day after calving.

Citrate

As with the concentration of AC+pdACAC, the highest but quickly and gradually decreasing concentration of milk citrate was found in the first 4 weeks of lactation. Citrate reached its minimum level (approx. 2 mmol/l) around the 40th day after calving (Fig. 3). After this minimum citrate slightly increased, but even in the 3rd month its level was only about 30% of the basal value.

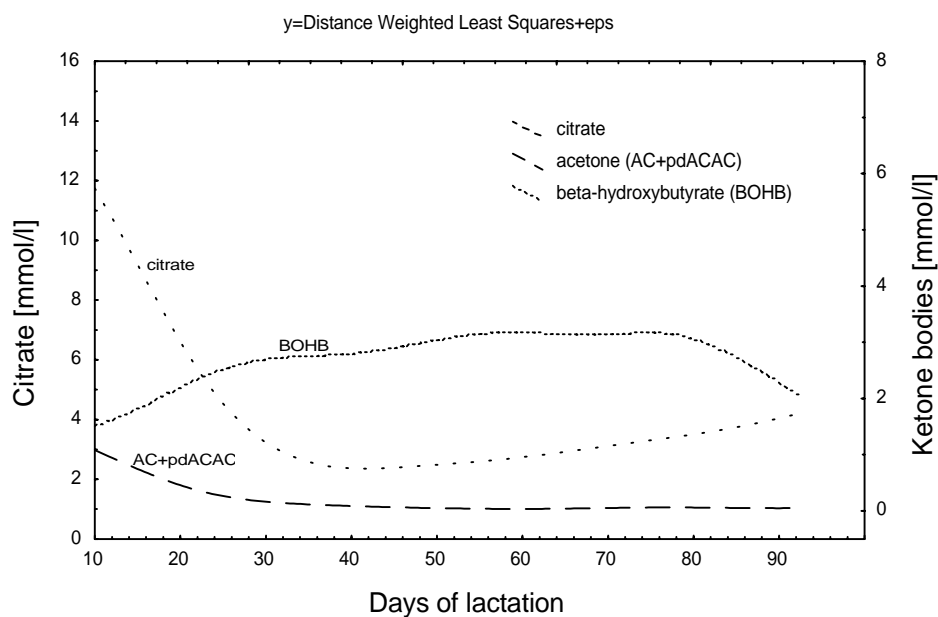


Fig. 3. Concentration of ketone bodies and citrate in milk plotted against the days of lactation (n = 119). AC = acetone, AC+pdACAC = measured acetone concentration, dACAC = previously decarboxylised acetoacetate, BOHB = β -hydroxybutyrate

Correlations between ketone bodies and citrate are shown also in Table 1. Citrate expressed a negative correlation with BOHB in both groups and, which is more important, a positive correlation with AC+pdACAC in the HA group.

Discussion

The distribution of ketone bodies in biological fluids is important in the detection of increased ketogenesis in dairy cows. Urine contains the highest concentration of ketone bodies, whereas the blood plasma and especially the milk contains lower levels (Schultz and Myers, 1959; Gaál, 1999).

Unfortunately, it has not been clarified how the three ketone bodies are distributed in the milk of healthy and ketotic cows, most probably due to the absence of unified analytical methods. In the blood serum of healthy mammalian species the following relative proportions are generally accepted: 78% β -hydroxybutyrate, 20% acetoacetate and 2% acetone (Burtis and Ashwood, 1999). Exact data on relationships among the three ketone bodies in milk are not available. In this study we observed a reverse distribution of acetoacetate and acetone in milk compared to their distribution in the blood, published by Schultz and Myers (1959). At 4 °C and at room temperature (20 °C) acetoacetate in the sample is unstable and disappears at rates of 6% per day and 6% per hour, respectively (Työppönen and Kauppinen, 1980). This phenomenon most probably explains our observation that in the 48-h-old milk samples only very low level of acetoacetate was found, but further investigations are needed to clarify this establishment. Consequently, the measured acetone concentrations contained the majority of the amount of acetoacetate present in the sample.

The relationship found between ketone bodies and citrate in milk during lactation meets the data of other authors (Illek et al., 1997; Khaled et al., 1999). The relatively high citrate concentration found in healthy cows about 10–20 days after calving can be explained by the intensive TCA cycle at that time. One of the key factors in both ketogenesis and citrate formation (TCA cycle) is oxaloacetate. As lactation advances, oxaloacetate is used mainly for gluconeogenic processes and the apparent lack of oxaloacetate can also explain the simultaneous decrease of citrate and increase of ketone bodies. There are some other milk constituents, such as milk protein, that also tend to decrease with advanced lactation (Rossow et al., 1990; Souci et al., 1994).

Nearly all of the HA samples came from the early stage (first 2–4 weeks) of lactation. As that period is critical in the development of metabolic diseases associated with energy deficiency (fatty liver, ketosis), we focused on the results of the HA group. The initial high level and the parallel drop of acetone (AC+pdACAC) and citrate in the milk of this group in the first few weeks of lactation is remarkable. We think that the regular, simultaneous control of their concentration in the milk could be used in monitoring the energy status of dairy cows, and it would make the evaluation of energy status easier and more certain. The determination of acetone and citrate can be easily automated. Following the complete development of these simple, easy methods they should be introduced in the routine laboratory analysis of milk as soon as possible. Automation of

acetone determination via flow injection analysis (FIA) is already approved in the dairy industry as validated methods are available (Baticz et al., 2001). In addition, introducing an automated citrate determination via Fourier transformation infrared analysis (FTIR) or FIA in cow's milk could be an additional analytical tool to find out or confirm early energy deficiency.

This monitoring system does not require extra milk sampling. Measurements can be carried out on milk samples monthly sent to milk laboratories for other purposes.

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