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An Automatic Flow Procedure for the Determination of 3-Hydroxybutyrate in Animal Serum and Plasma

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An automatic flow procedure based on the multicommutation concept, comprising three-way solenoid valves, for the spectrophotometric determination of 3-hydroxybutyrate in animal serum and plasma is proposed. The 3-hydroxybutyrate was enzymatically converted to acetoacetate with the reduction of NAD^+ to NADH monitored at 340 nm. It was possible to carry out up to 600 determinations without a significant decrease in the analytical signal, with 5 mg of 3-hydroxybutyrate dehydrogenase immobilized on porous silica beads and packed in a column. The system enabled 60 determinations/h of 3-hydroxybutyrate in the range of 10−150 mg L^{-1}, with a consumption of 0.9 mg of NAD^+ and 200 μL of sample per determination. A detection limit of 2 mg L^{-1} for both animal serum and plasma and coefficients of variation of 1.4% and 1.2% ($n = 17$), respectively, were determined. Animal serum and plasma samples were analyzed without previous treatment, the results of which agreed with those obtained using the conventional method (UV kit, Sigma).

KEYWORDS: Flow analysis; multicommutation; 3-hydroxybutyrate; spectrophotometry; enzyme reactor; plasma and serum

INTRODUCTION

Within the tropical and subtropical regions, the general level of ruminant nutrition frequently experiences a deficit, mainly in the dry period, thus requiring diet supplementation. The supplemental feed has been shown to improve the quality of the animal diet (*1*). In an indirect way, the nutritional deficiency can be reflected in the chemical composition of the blood (*2*). Considering this effect, Payne and Payne (*3*) suggest the elaboration of a metabolic profile to control the nutritional condition of the animals by the determination of the representative biochemical species in the animal blood. In this way, it should be possible to investigate the relationship between feeding and representative sanguine parameters on animal performance (*2*).

The evaluation of the absorption efficiency of the nutrients administrated in the animal diets can be periodically accomplished by using blood analysis results of substances produced by the metabolism of the animal (*4*, *5*), and among them, 3-hydroxybutyrate is cited (*6*).

3-Hydroxybutyrate (HB) is an important metabolite in the metabolism of glucose (*6*, *7*), and in plasma and serum, it reflects the balance between fat mobilization and the animal's capacity to utilize the ketone bodies produced (*8*). Reference values for HB up to 118 mg L^{-1} for animals in lactating condition and 32 mg L^{-1} for those nonlactating are usually accepted as normal.

Under starvation conditions, HB concentrations in blood are higher than reference values, indicating an inadequate carbohydrate intake and ketonemia. As a consequence, this can causes decreased milk production and diseases such as hypophagia and lethargy (*8*, *9*). In this condition, subclinical ketosis is also common and encompasses mild hyperketonemia and lower concentrations of blood glucose (*10*, *11*). Therefore, the HB concentration of animal blood is a parameter that can be used to follow the health and nutritional status of the animal.

The most commonly used methods for HB determination are those based on enzymatic reaction (*7*, *12*), offering advantages over the existing procedures based on the oxidation of HB to acetone with subsequent spectrophotometric determination (*13*).

In general, studies on animal metabolism require the analysis of several samples, and the manual methods are laborious. Therefore, an automatic procedure would provide a more practical, user-friendly approach.

In this paper, an automatic multicommutated flow system with spectrophotometric detection for the determination of HB in animal serum and plasma is reported, employing the hydroxybutyrate dehydrogenase enzyme immobilized on a bead reactor. This system is aimed at decreasing reagent and sample

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consumption and sample manipulations, increasing the analytical frequency, and enhancing the precision of measurements.

MATERIALS AND METHODS

Apparatus. The system consisted of a 700 Plus Femto spectrophometer with a 80-μL flow cell (optical path = 1 cm), a peristaltic pump (Ismatec, IPC-4, Glattbrugg, Switzerland) equipped with Tygon pumping tubes, and a microcomputer furnished with an electronic interface card (Advantech Corp. PCL-711S, San Jose, CA). The computer-controlled flow system was assembled with three-way solenoid valves (Nresearch-161T031, W. Caldwell, NJ), mixing coils of polyethylene tubing (0.8-mm i.d.) and Perspex joint devices. To switch on these valves, a potential difference of 12 V was supplied by a homemade electronic interface (*14*). Software written in Quick BASIC 4.5 was used to control the flow system and perform data acquisition.

Reagents and Solutions. All chemicals were of analytical grade, and purified water (conductivity less than 0.1 μS cm^{-1}) obtained with a Mille-Q system was used throughout. Sample and standard solutions were stored in polyethylene bottles.

The HB stock solution (500 mg L^{-1}) was prepared by dissolving the corresponding sodium salt (Sigma Chemical Company, St. Louis, MO) in water. Standard solutions of 0.0, 10.0, 25.0, 50.0, 75.0, 100.0, 125.0, and 150.0 mg L^{-1} HB were prepared in water by appropriate dilutions from the stock solution and were stored in a refrigerator when not in use. A 7.0 mmol L^{-1} NAD$^+$ solution (Sigma, Grade AA-1, 90%, St. Louis, MO) was prepared daily in buffer solution (pH 9.3) consisting of 0.1 mol L^{-1} glycine plus 0.1 mol L^{-1} NaCl and 0.1 mol L^{-1} NaOH. A 0.1 mol L^{-1} phosphate buffer solution (pH 7.0) was employed as a carrier stream. Considering that this solution is the one that remained in contact with the immobilized enzyme for a longer period of time, a pH value similar to that of the conditioning solution of 3-hydroxybutyrate dehydrogenase (HBDH) was used.

The HBDH enzyme (5 mg) was immobilized on aminopropyl porous silica beads (0.1 g), pore size 170 Å and 200−400 mesh (Sigma, St. Louis, MO), according to the procedure described by Kiba et al. (*7*). The aminated beads were packed into a cylindrical column (1 cm × 3 mm i.d.) using a syringe. Prior to immobilizing the enzyme, the aminated beads were conditioned by pumping a glutaraldehyde solution (2% w/v) in a 0.1 mol L^{-1} phosphate buffer (pH 7.0) through the column for 3 h and maintaining the temperature at 25 °C. Thereafter, the column was washed with deaerated water for 30 min. The enzyme solution was prepared using 5 mg (600 U, 1.0 mL) of HBDH (Rhodobacter Spheroids, Boehringer Ingelheim GmbH, Germany) diluted to 10 mL using a 0.05 mol L^{-1} phosphate buffer (pH 7.0) solution. The enzyme immobilization on the aminated beads was carried out by pumping its solution through the column in a closed loop for 6 h and maintaining the temperature at 25 °C. An assay carried out with the remaining solution after this period showed that no enzymatic activity was detected, thus indicating that the enzyme was well immobilized.

When not in use, the column was maintained in a 0.1 mol L^{-1} phosphate buffer solution (pH 7.0) at 4 °C in the refrigerator. Under these conditions, it could be used for at least 6 months.

Sample Preparation. The serum and plasma samples were collected directly from the animals (male sheep and cows) as recommended in the literature (*15*). The blood was collected in tubes with anticoagulant and centrifuged to 2500 rpm for 12 min. The plasma was removed and transferred to another tube. In the case of serum, anticoagulant was not used. After collection, the sample (10 mL) was allowed to rest for 24 h to allow for clotting and separation of the serum. A serum aliquot (4 mL) was transferred to another tube, while the coagulated blood was centrifuged to obtain a more appropriate separation (*8*). The samples could be maintained in the freezer for long periods of time, although before analysis, they had to be equilibrated to room temperature.

Flow System and Procedure. The flow network based on the multicommutation approach was designed using three-way solenoid valves. Its flow diagram is depicted in **Figure 1**. In this configuration, all valves are switched off, and only the carrier solution (C) is flowing through the analytical path (coils B_1, B_2, HBDH column) toward the detector (D), while the sample (S) and reagent (R) solutions are recovered. The aliquots of sample and reagent solutions are introduced in the analytical path through the confluence x and y by switching on valves V_1, V_2, and V_3 during time intervals previously defined. For the serum analysis, the three valves were switched on at the same time for a period of 10 s. While this step was followed, the carrier solution was recycled, and reagent (NAD$^+$) and 200-μL sample solutions were pumped toward confluences x and y. Thereafter, all valves were switched off, and the carrier solution flowed again through the analytical path and displaced the sample and reagent zone toward the detector. Assays were carried out changing the flow of reagent (NAD$^+$) solution from 0.7 to 1.4 mL min^{-1} and maintaining the flow rates of carrier and sample solutions streams at 1.4 and 1.2 mL min^{-1}, respectively.

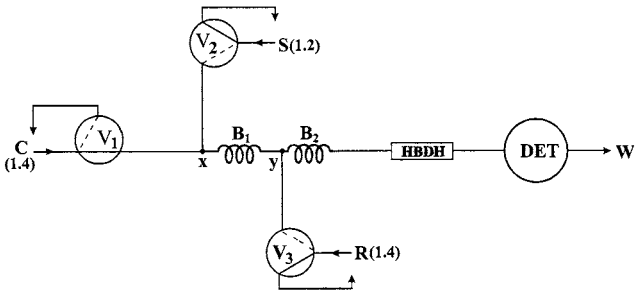

Figure 1. Flow diagram of the system. V_1, V_2, and V_3, three way solenoid valves; S, sample, flow rate at 1.2 mL min^{-1}; C, carrier stream (phosphate solution); R, NAD$^+$ reagent; x and y, confluence; B_1 and B_2, coiled reactors (15 and 30 cm, respectively), 0.8 mm i.d.; HBDH, enzyme column; DET, spectrophotometer detector (λ = 340 nm); W, waste. Arrows indicate flow direction. Numbers between brackets represent flow rates in mL min^{-1}. Solid and dashed lines in the valves symbol indicate fluid pathway when valves are switched off and on, respectively.

For plasma analysis, the valves were switched on at the same time. Therefore, after a delay time of 3 s, valves V_1 and V_2 were switched off. In this way, the carrier solution flowed through the analytical path, diluting the 60-μL sample before valve V_3 was switched off. Under this condition, the volume of sample aliquot merged into the enzymatic column (HBDH) was 66% lower than that used when serum was analyzed.

The quality of the results obtained with the developed automatic system was evaluated by comparing the values obtained in the analysis of 12 serum and plasma samples with the results supplied by the conventional method (Sigma UV kit). This method is based on the enzymatic conversion of HB catalyzed by HBDH to acetoacetate and reduced nicotinamide−adenine dinucleotide (NADH). The increase in absorbance at 340 nm due to NADH formed was directly proportional to the HB concentration in the samples.

RESULTS AND DISCUSSION

The first parameters studied were NAD$^+$ flow rate and its concentration. Better results were obtained with the flow rate at 1.4 mL min^{-1} and a 7 mmol L^{-1} NAD$^+$ solution. This concentration was similar to that found in the literature (*7*). Experiments were carried out using NAD$^+$ prepared in glycine solution, yielding results identical to those obtained using separate solutions (an on-line mixture of the glycine solution with NAD$^+$ prepared in phosphate buffer), as suggested by Kiba et al. (*7*). Considering these results, NAD$^+$ was prepared only in glycine solution to carry out the further experiments. Under this condition, the system manifold was simplified by reducing it to a single flow line.

Effect of pH and Temperature. The influence of the pH on enzymatic activity was evaluated by changing this parameter of the glycine solution in the range of 7.5−11.0 units, yielding the results shown in **Figure 2**. As can be seen, the pH has a remarkable effect on signal. Therefore, to ensure measurement stability, the glycine solution was buffered at pH 9.3.

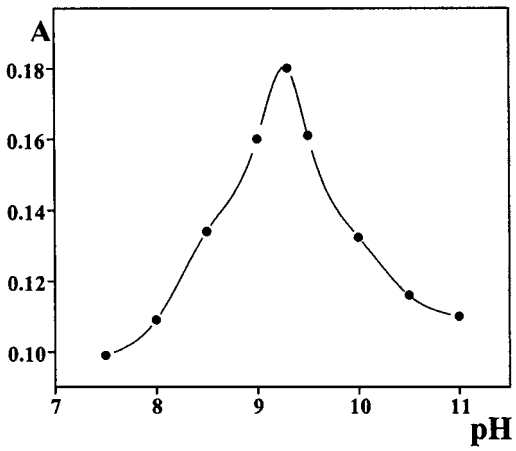

Figure 2. Effect of pH on the activity of the immobilized enzyme.

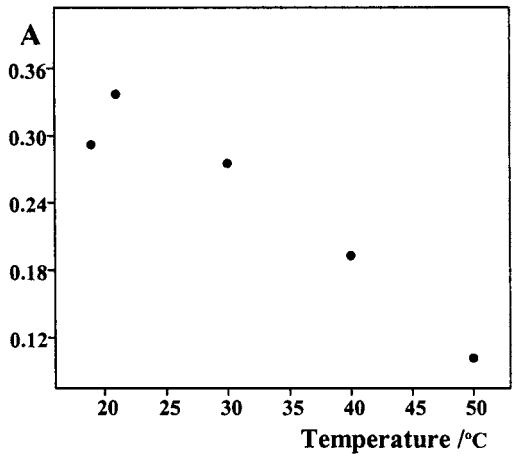

Figure 3. Effect of temperature on the activity of the immobilized enzyme.

The temperature effect on the enzymatic reaction was determined by varying the former from 19 to 50 °C. The maximum signal (**Figure 3**) was obtained for values near room temperature. Consequently, the use of a thermostatic bath was discarded for further experiments.

Sample Analysis. The plasma samples presented viscosities higher than serum samples. This feature increased the backpressure of the enzyme column, causing fluid leakage in the flow line connections. It was verified that diluting the sample three times was sufficient to overcome this obstacle; thus, an on-line dilution step that was done by switching on valve V_2 (**Figure 1**) for 3s to insert a sample aliquot of 60 μL into coil B_1 was programmed into the control software, while for serum analysis, the time duration was 10 s to insert a sample volume of 200 μL.

The chemical conditions recreated in the flow system were similar to those used with the same enzymes in the conventional procedure, and as in this procedure, there were no interferences from other species present in the matrixes (recovery tests carried out did not present values that deviated significantly from 100%).

The analytical curves of both the serum and plasma samples were linear in the concentration range 10 to 150 mg L^{-1} HB presenting the following relationship $y_s = 0.0881 + 0.0039x_s$; ($r = 0.9988$) for serum and $y_p = 0.0562 + 0.0012x_p$ ($r = 0.9978$) for plasma, where y and x were analytical signals expressed in absorbance units (AU) and analyte concentrations in mg L^{-1} HB, respectively.

Table 1. Results Obtained in the Determination of 3-hydroxybutyrate in Serum[a] and Plasma[b] Samples[c] by the Proposed System and the Sigma UV–Kit

sample	proposed system	Sigma UV kit
1[a]	19.8 ± 0.0	18.4 ± 0.8
2[a]	23.6 ± 0.5	23.4 ± 1.5
3[a]	19.1 ± 0.9	20.1 ± 2.9
4[a]	22.7 ± 0.0	22.4 ± 1.1
5[a]	69.7 ± 1.8	67.0 ± 0.6
6[a]	69.8 ± 0.6	68.3 ± 0.6
7[a]	63.7 ± 1.9	65.8 ± 1.4
8[a]	36.8 ± 0.9	35.1 ± 1.0
9[b]	46.0 ± 0.3	50.0 ± 0.0
10[b]	44.1 ± 0.2	40.0 ± 0.0
11[b]	50.1 ± 0.1	50.0 ± 0.0
12[b]	45.1 ± 0.3	50.0 ± 0.0

[c] Concentration in milligrams per liter (mean values and uncertainties, $n = 3$).

The long-term stability of the HBDH enzyme immobilized into the minicolumn was verified by carrying out assays periodically using a HB standard solution. When not in use, the minicolumn was stored in a 0.1 mol L^{-1} phosphate buffer (pH 7.0) solution at 4 °C. Under these conditions, and after 6 months of storage, a decrease in signal of ~5% was observed. In this sense, one can consider that HBDH enzyme immobilized in the minicolumn is very stable, permitting its use as a recently prepared column for at least 6 months. On the other hand, working continuously on a daily basis, which always began with a calibration procedure, 600 determinations could be carried out with a decrease in sensitivity <5%.

The feasibility of the system was verified by performing the HB determination in a set of serum and plasma samples, yielding the results summarized in **Table 1**. Accuracy was assessed by applying the Student's *t*-test to the results through carrying out a bilateral coupled test (paired *t*-test) (*16*), with those obtained by the conventional method (Sigma UV kit). Once the calculated value of $|t|$ was less than the critical value tabulated, no significant difference was observed at a 95% confidence level. Other profitable characteristics, such as a sample throughput of 60 determinations/h; a detection limit of 2 mg L^{-1} HB obtained with a blank solution (three times the signal blank/slope, $n = 30$) at 99.7% confidence level for both samples (*17*); a relative standard deviation of 1.4%, 1.2% ($n = 17$) for typical samples of serum and plasma containing 75 mg and 50 mg L^{-1} HB, respectively; and low consumptions of NAD^+ (0.9 mg) and HBDH enzyme (8 μg) per determination, were also achieved.

The proposed system is very simple and easy to operate and can be implemented using inexpensive instrumentation with facilities to carry out data acquisition and automatic dilution. The system presented enables the determination of HB with just HBDH and NAD^+, without the need for another enzyme or other reagents (*18*, *19*). It also has the advantage of giving rise to a stability ~6 months of HBDH immobilized enzyme. The sample throughput (60 determinations/h) was at least twice that of existing flow analysis procedures (*7*, *18*, *19*), while the consumption of enzyme per analysis was lower than that related to other procedures (*7*, *18*, *19*). If we consider the conventional method used, in addition to economy of enzyme used, (1U per determination in the proposed procedure against 5U in the UV kit), the consumption of NAD^+ is reduced to one-tenth, and the results of each determination are obtained in a shorter time span (1 min, as compared to 15 min in the UV kit).

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