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Development of A Rapid Microtiterplate Based Colorimetric Method for Estimation of Non-esterified Fatty Acids in Bovine Plasma

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Abstract

Non-esterified fatty acid (NEFA) is one of the most important blood metabolites contributing significantly during body growth, cyclicity, pregnancy and lactation, and it plays more vital role particularly during transition phase of the biological processes like pregnancy to lactation transition period. Determination of blood concentrations of NEFA is, therefore, important. The measurement technique or assay for measurement of blood NEFA should be simple, reliable, accurate, rapid and cheap. Here, the authors developed a sensitive and fast microtiterplate colorimetry-based method for estimation of NEFA in bovine plasma. The method is simple to perform and gives accurate, precise results in 100 µL samples. The developed method requires at least five times less quantity of plasma and reagents than that of already existing methods. Furthermore, ability of the method for taking reading of all the samples together on microtiterplate in a reader enable us to save a lot of time. Hence, the developed method may be adopted for estimation of NEFA in bovine plasma.

Keywords: *NEFA, bovine, microtiterplate, plasma, assay*

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INTRODUCTION

Blood metabolites are important constituents of bovine plasma/serum and certain of them play crucial role during the process of growth and productive stages like reproductive cycle, pregnancy and lactation. Non-esterified fatty acid (NEFA) is one of the most important blood metabolites contributing significantly during body growth, cyclicity, pregnancy and lactation, and its role becomes vibrant particularly during transition phase of the biological processes like pregnancy to lactation transition period [1–3].

A massive mobilization of NEFA from adipose tissue during and after parturition in high-yielding dairy cows is the metabolic hallmark of the transition from pregnancy to lactation [4]. There is an abrupt shift in the metabolic demands from nutrient accrual (body reserves and fetal mass) to rapid mobilization of lipid and protein stores in support of the sudden onset of high milk production [5, 6]. In addition to the demand of high energy owing to high milk production, decreased dry matter intake (DMI) during the peri-parturient period leads to reduced energy intake prior to calving. Hence, the cows experience some degree of negative energy status [7] especially during the final week before calving [8]. Then, a phenomenon known as negative energy balance (NEB) occurs in transition dairy cows when energy requirements (needed for milk production) exceed energy intake [9]. Ultimately, NEB results in mobilization of fats/lipids from the adipose tissues and also from other internal organs in extreme conditions, and due to mobilization of fatty acids from various tissues/organs to bloodstream causes rise in the concentrations of fatty acids in blood. The NEFA are converted to more available energy substrates once the liver transforms them into ketone bodies. The most common ketone body in dairy cows is beta hydroxybutyrate (BHB). This ketone body is used by muscle and

nervous tissue as an energy substrate, but in excess causes clinical problems, for example, ketosis. Traditionally, NEFA and BHB were used to assess the degree of NEB and lipid mobilization. They also can be used to evaluate the performance of transition cow nutrition programs. Feeding strategies designed to increase energy intake during this period are desirable for optimal postpartum health and performance. Thus, monitoring of plasma NEFA is a useful tool for dry cow management. The measurement of nonesterified fatty acids (NEFAs) in dairy cows can be used to assess dry cow nutritional management. Determination of blood concentrations of NEFA is, therefore, important.

The measurement technique or assay for measurement of blood NEFA should be simple, reliable, accurate, rapid and cheap. A large number of methods for detection and isolation of NEFA have been previously reported. A copper soap extraction method of Shipe *et al.* [10] of estimating NEFA in milk, and thereafter modified method of Shipe's for measuring blood NEFA have been used widely in different laboratories in India [1–3]. Though the modified methods as mentioned above are accurate and reliable, it requires large quantity of plasma or milk (0.5 mL of milk or plasma in duplicates), and also takes a lot of time for taking final spectrophotometric reading particularly when a large number of samples are to be used for estimation of NEFA as reading has to be taken individually or as a group in a cuvette for each sample. It would be advantageous if the assay procedure needs minimum quantity of valuable plasma sample and also requires less time for taking the final reading using spectrophotometer. Therefore, the objective of the present study was to develop a method of estimating NEFA in bovine plasma based on microtiter plate colorimetry. The authors compared their newly developed technique for estimation of plasma NEFA with that of modified Shipe's method.

MATERIALS AND METHODS

Chemicals

Triethanolamine, cupric nitrate [$Cu(NO_3)_2.3H_2O$], hydrochloric acid, sodium chloride (NaCl), sodium hydroxide (NaOH), sodium diethyl dithiocarbomate, n-butanol, chloroform, heptane and methanol and palmitic acid. All chemicals were procured from Merck Bioscience (India) unless otherwise stated.

Reagents

Copper Reagent

A mixture of 5 mL of triethanolamine and 10 mL of 1 M aqueous cupric nitrate [$Cu(NO_3)_2.3H_2O$] was diluted to 100 mL with saturated sodium chloride (NaCl) solution. The pH was adjusted to 8.3 with 1 N sodium hydroxide (NaOH) solution. The mixture was stored in a dark at room temperature, in order to ensure that the material remained stable for a period of at least 4–5 months.

Color Reagent

0.5% sodium diethyl dithiocarbomate solution in n-butanol, i.e., 0.5 g per 100 mL.

Solvent Mixture

Chloroform, heptane and methanol (all GR grade) were mixed in a proportion of 49:49:2, respectively, and the mixture was designated as CHM.

Preparation of Standard

The standards were prepared with palmitic acid as specified by Koops and Klomp [11]. In brief, 0.2 M solution of palmitic acid was prepared in solvent mixture as described above. 1 mL of this stock solution was diluted to 100 mL with the solvent mixture giving the final concentration of 2000 µmol/L. Standards of different concentrations, viz., 0.00, 31.25, 62.50, 125, 250, 500, 1000 and 2000 µmol/L were run in the assay. The results were expressed as µmol NEFA/L of plasma.

Methods

For the development of the method for estimation of NEFA in bovine plasma, the authors estimated NEFA in all the samples using the method as described by Shipe *et al.* [10] and also using the method developed by the authors. The results obtained were compared between the methods.

Shipe's Method

A 0.1 mL aliquot of 0.7 N HCl was added to 0.5 mL plasma/standard in a 16×125 mm screw cap test tube. The mixture was shaken

on a vortex test tube mixer and 2 mL of the copper reagent and 6 mL of the solvent mixture were added. The contents were then shaken for 30 min on a shaker at 240 rpm and then centrifuged for 10 min at 3000 rpm. 3.5 mL of the solvent layer was transferred to an acid washed test tube containing 0.1 mL of the color reagent. After mixing, the color intensity was measured within 1 h at 440 nm using spectrophotometer against blank, prepared in the same manner and using 0.5 mL double distilled water in place of plasma/standard. The content of non-esterified fatty acid was calculated from the standard curve.

Method Used

A 20 µL aliquot of 0.7 N HCl was added to 100 µL plasma/standard in a 2-mL glass test tube. The mixture was shaken on a vortex test tube mixer and 400 µL of the copper reagent and 1.2 mL of the solvent mixture were added. The contents were then shaken for 30 min on a shaker at 240 rpm and then centrifuged for 10 min at 3000 rpm. 200 µL of the solvent layer was transferred directly in the wells of 96-well microtiter plate containing 20 µL of the color reagent. After mixing, the color intensity was measured within 1 h at 440 nm using microtiter plate reader (Model: ECIL, Microscan, India). The content of non-esterified fatty acid was calculated from the standard curve.

Sample Analysis

A total of 20 samples were assayed for plasma NEFA using both the methods as described above.

Statistical Analysis

All data were expressed as mean±S.E.M. Standard regression analyses were used to determine the statistical significance of correlations between the NEFA concentrations estimated by the developed method and Shipe's method [10], and NEFA added and recovered. Significance was considered at $P<0.05$ level, if otherwise not stated. GraphPad Prism 4.01 software (San Diego, USA) was used for statistical analysis of the data.

RESULTS AND DISCUSSION

Standard Curve Preparation Using Shipe's Method

Standards of different concentrations, viz., 0.00, 31.25, 62.50, 125, 250, 500, 1000 and 2000 µmol/L prepared as described in the materials and method section were run in an assay**.** An amount of 0.5 mL of standard in duplicate was used. The optical density of around 2.0 at 440 nm with the highest concentration tested, i.e., 2000 µmol/L was observed. The standard curve showed linearity (Figure 1). The sensitivity of the assay was found to be 31.25 µmol/L.

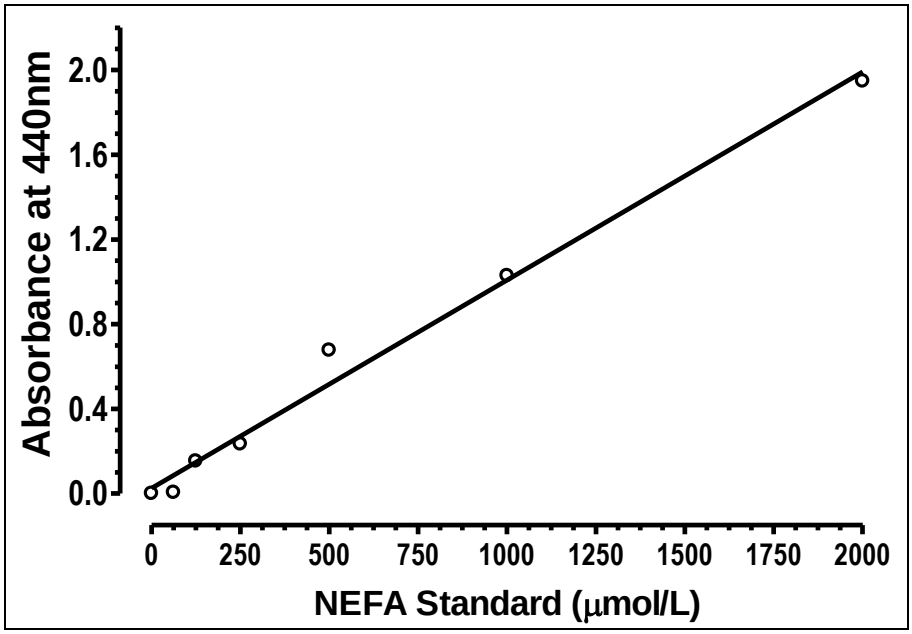

Fig. 1: *Standard Curve of NEFA in Shipe's Method.*

Standard Curve Preparation Using the Developed Method

An assay was run with same standards as used in the Shipe's method but with 1/5th less volume, i.e., 100 µL. It showed an optical density of around 1.0 at 440 nm with the highest standard (2000 µLmol/L) used in the assay. The standard curve showed linearity (Figure 2). The lowest level of detection limit (sensitivity) of the assay was 31.25 µLmol/L.

Recovery of Known Quantity of Added NEFA

Recovery of the known amount of added NEFA (25, 50, 100, 150, 200, 250, 300, 400, 500, 750, 1000 and 2000 µLmol/L) was determined using the method as described herein. Recoveries (mean±SEM) were 23.2±2.27, 52.6±2.31, 98.3±2.71, 147.5±2.73, 203.13±3.19, 245.2±4.35, 297.4±3.31, 396.7±3.93, 489.96±7.87, 742.5±6.45, 996.5±4.63 and 1991.2±7.95 µLmol/L, respectively. The correlation coefficient of the added and recovered NEFA concentrations was significant (r=0.99; P<0.001). Recovery of added (X) versus recovered (Y) NEFA concentrations was described by linear regression (Figure 3) and gives the equation Y=0.9954+1.005X.

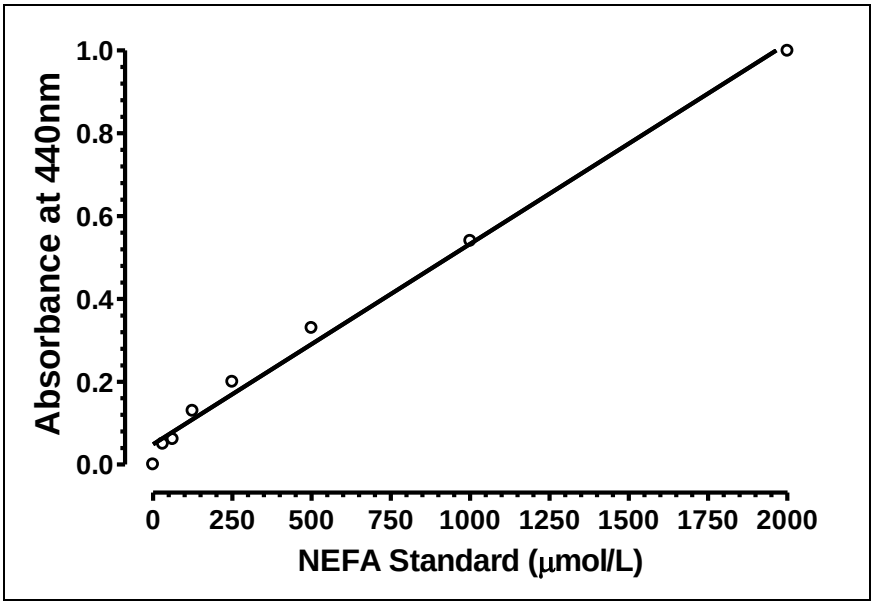

Fig. 2: *Standard Curve of NEFA in Authors' Developed Method.*

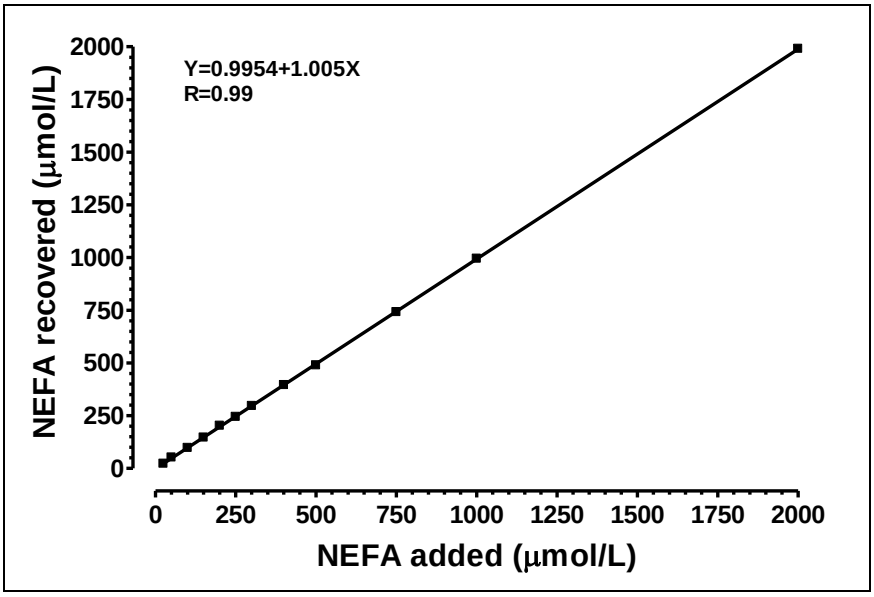

Fig. 3: *Linear Regression of Recovery of NEFA Added and Measured in the Assay.*

Measurement of Plasma NEFA Using Shipe's *vis-à-vis* the Newly Developed Method

The concentrations of NEFA were measured in 20 bovine plasma samples using the developed methods as described in the present study. The results were compared with the concentrations of NEFA obtained by using the method of Shipe [10] in the same samples. The correlation of coefficients between the methods was significant (P<0.0001; r=0.99). Concentrations of NEFA as measured with the developed method (Y) versus Shipe's method (X) was described by linear regression (Figure 4) and gives the equation Y=0.9996+0.9657X.

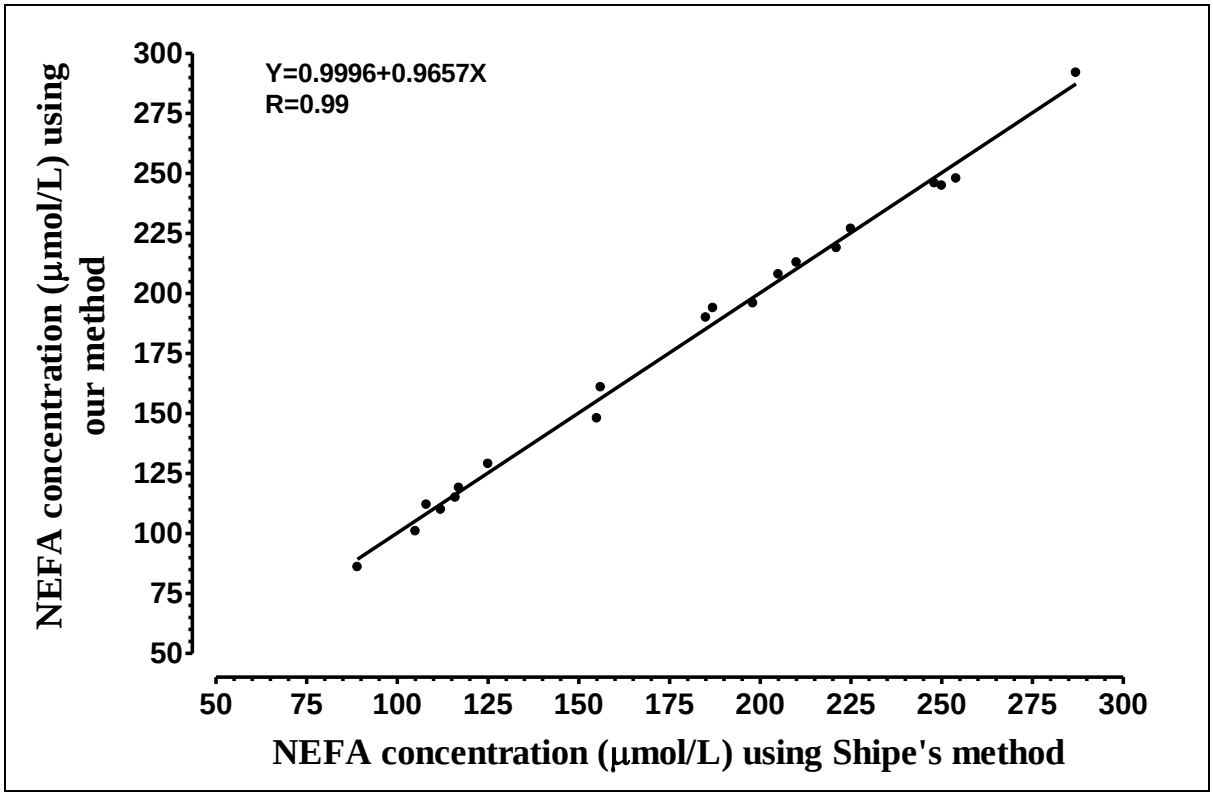

Fig. 4: *Correlation between NEFA Concentrations Measured in 20 Bovine Plasma Samples by Using the Developed vis-a-vis Shipe's Method. The Correlation Was Significant (P<0.001).*

To the best of the authors' knowledge, this is the first report on developing a method based on microtiterplate colorimetry for estimation of NEFA in bovine plasma. The developed method as described here has similar sensitivity and estimates same concentrations of NEFA in bovine plasma as that of the method of Shipe [10]. However, the method as developed in the present study has several advantages over the existing method of Shipe [10] for plasma NEFA estimation: (a) it needs several times less plasma volume (five times) thereby saves valuable plasma sample; (b) the reagents required for the estimation of NEFA are also much lesser and (c) a number of samples can be read at time in a microtiterplate reader instead of taking reading of individual sample each time thereby making the method faster.

CONCLUSIONS

In conclusion, the authors have developed a sensitive, reliable, fast and cheap method for estimation of NEFA in bovine plasma for the first time using microtiterplate based colorimetry. The developed method requires at least five times less quantity of plasma and reagents than that of already existing method of Shipe [10]. Furthermore, ability of the method for taking reading of all the samples together on microtiterplate in a reader enable us to save a lot of time. Hence, the developed method may be adopted for estimation of NEFA in bovine plasma.

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