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Determination of Bovine Serum Low-density Lipoprotein Cholesterol using the N-geneous Method

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ABSTRACT

The N-geneous method is a recently developed method for determination of low-density lipoprotein cholesterol (LDL-C) in human serum. In the present study, we attempted to adapt this method to bovine serum. The values of LDL-C obtained using the N-geneous method were highly correlated with those from the method using ultracentrifugation and heparin sepharose affinity chromatography ($r = 0.934$, $p < 0.001$). The reproducibility of this method was acceptable (intra-assay CV 4.2%, inter-assay CV 7.6%) for clinical use. Using the N-geneous method, serum LDL-C was evaluated in cows around parturition, and in cows with fatty liver induced by fasting. The concentration of LDL-C decreased significantly in cows close to parturition. A reduced concentration of LDL-C was also observed in cows with fatty liver. In both cases, the changes of LDL-C were similar to those of apolipoprotein B (apoB)-100, and the values of LDL-C were highly correlated ($r = 0.876$, $p < 0.001$) with those of apoB-100. These results suggest that the concentration of LDL-C reflects the level of apoB-100. The N-geneous method is simple and rapid, and might to be a useful tool to elucidate the clinical significance of LDL-C in bovine serum.

Keywords: apolipoprotein B-100, cattle, cholesterol, low-density lipoprotein, low-density lipoprotein cholesterol

Abbreviations: apoB, apolipoprotein B; HDL, high-density lipoprotein; HDL-C, high-density lipoprotein cholesterol; LDL, low-density lipoprotein; LDL-C, low-density lipoprotein cholesterol; NEFA, non-esterified fatty acid; SRID, single radial immunodiffusion; TC, total cholesterol; TG, triglyceride; VLDL, very low-density lipoprotein

INTRODUCTION

Low-density lipoprotein (LDL) is one of the cholesterol-rich lipoproteins and its major protein component is apolipoprotein B (apoB)-100. LDL is recognized to be an end product of the intravascular degradation of chylomicron and very low-density lipoprotein (VLDL). The main function of LDL is distribution of cholesterol to peripheral tissues, mediated by the LDL receptor (apoB,E receptor). In human plasma, LDLs are the predominant plasma lipoproteins, whereas bovine LDLs account for less than 10% of total lipoproteins in plasma and intestinal lymph (Bauchart, 1993).

It is well established that LDL-cholesterol (LDL-C) is one of the most important parameters for estimating hypercholesterolaemia, which is a risk factor for atherosclerosis

in humans. Although bovine plasma apoB-100 is useful for diagnosis of fatty liver and related diseases in dairy cows (Marcos *et al*., 1990a; Oikawa *et al*., 1995; Oikawa and Katoh, 1997, 2002), the clinical significance of bovine LDL-C is unknown. This is probably because of the difficulty of measurement, in addition to its low concentration in plasma.

The density range of bovine LDL particles is 1.026–1.076 g/ml, which is wider than that of humans. Bovine high-density lipoprotein (HDL) also has a wide density range, and is generally present in plasma as two distinct main populations: light HDL (1.060–1.091 g/ml) and heavy HDL (1.091–1.180 g/ml). Moreover, very light HDL (1.039–1.060 g/ml) has also been identified in plasma (Bauchart *et al*., 1989; Bauchart, 1993). Since the density ranges of light HDL and very light HDL overlap that of LDL, satisfactory discrimination between bovine LDL and HDL requires heparin-Sepharose affinity chromatography, in addition to ultracentrifugation (Cordle *et al*., 1985).

In human serum, LDL-C is currently derived by the Friedewald equation, using the values of total cholesterol (TC), HDL-cholesterol (HDL-C) and triglyceride (TG): LDL-C = TC−(HDL-C−TG/5) (Friedewald *et al*., 1972). HDL-C is usually determined by the selective precipitation method, which is based on the specific interaction of apoB with a precipitating agent such as dextran sulphate-Mg^{2+} or heparin-Mn^{2+}(Burstein and Scholnick, 1973). After apoB-containing lipoproteins (LDL, VLDL) are precipitated, HDL-C is determined by measuring cholesterol in the supernatant. In a preliminary study, however, we confirmed that bovine serum HDL-C determined by the dextran sulphate-Mg^{2+} method was not correlated with that determined by ultracentrifugation. This was presumably because a part of light HDL, which had a size close to that of LDL, also precipitated together with LDL and VLDL.

Recently, a new method, the N-geneous method (Rifai *et al*., 1998), was developed for determination of LDL-C in human serum. However, there is no information on the adaptation of this method to bovine serum LDL-C. In the present study, we compared the N-geneous method with the method using ultracentrifugation and heparin-Sepharose affinity chromatography (UC-HS method). Using the N-geneous method, serum LDL-C was evaluated in cows around parturition, and in cows with fatty liver induced by fasting.

MATERIALS AND METHODS

Samples

Blood samples were collected from 3 Holstein male calves (2 months old), 8 Holstein heifers (2–3 years old) and 12 lactating cows (3–4 years old, 80–150 days after parturition). The sera were used for determination of assay conditions in the N-geneous method, and for comparison between the methods for LDL-C determination. To investigate the changes of LDL-C around parturition (from 4 weeks before parturition to 10 weeks after parturition), sera were collected from 10 dairy cows at 2-week intervals. In addition, 4 non-lactating cows (4–6 years old) were used for induction of fatty liver by fasting. After 5 days of fasting, cows were re-fed for 7 days. Biopsy of livers was performed 10 days before fasting and 4 days after fasting. Blood samples were collected every day.

Determination of LDL-C by N-geneous method

Serum LDL-C was determined using a commercially available kit, Choletest LDL (Daiichi Pure Chemicals, Tokyo, Japan). Although this kit was conditioned for an automatic analyser, we used it by manual handling in this study. Briefly, the serum sample was incubated with Reagent 1 for 5 min at 37°C. After addition of Reagent 2, the reaction mixture was further incubated for 5 min at 37°C. The difference in absorbance between 660 nm and 546 nm was measured. The assay was calibrated using the Choletest LDL Calibrator (Daiichi Pure Chemicals). The principle of this method is as follows. After Reagent 1 is mixed with the serum, Detergent 1 specifically disrupts non-LDL lipoproteins and releases free and esterified cholesterol. Esterified cholesterol is hydrolysed to the free form by cholesterol esterase. Cholesterol oxidase catalyses free cholesterol and generates hydrogen peroxide, which is consumed by a peroxidase in the presence of 4-aminoantipyrine to generate a colourless product. On the addition of Reagent 2, Detergent 2 specifically releases cholesterol from LDL particles. A reaction similar to that described above occurs, but generated hydrogen peroxide reacts with *N,N*-bis-(4-sulphobutyl)-*m*-toluidine disodium salt to develop a coloured product.

Separation of lipoproteins by sequential ultracentrifugation

Serum lipoproteins were separated by sequential ultracentrifugation (Hatch and Lees, 1968), with slight modification. Ultracentrifugation was performed on a Himac CS 150GX ultracentrifuge (Hitachi, Tokyo, Japan), using a fixed-angle S100AT rotor, with 2.4 ml of bovine serum at the following densitied: very low-density lipoprotein (VLDL), $d < 1.006$ g/ml; LDL, $d < 1.063$ g/ml; HDL, $d < 1.21$ g/ml. In each step, samples were centrifuged at 604 000g, and 1.2 ml of the top fraction was collected. Centrifugation times for the VLDL, LDL and HDL separations were 2 h, 4 h and 8 h, respectively. After collecting HDL fraction at the final step, the bottom fraction (non-lipoprotein fraction, $d > 1.21$ g/ml) was also recovered. The obtained fractions were dialysed three times against 20 mmol/L phosphate buffer (pH 7.4) containing 0.05 mol/L NaCl and 0.02% sodium azide for 12 h at 4° C.

Heparin-sepharose affinity chromatography

LDL and HDL fractions were combined and applied to heparin-Sepharose columns (1 ml, Hitrap Heparin, Amersham Biosciences, Uppsala, Sweden), which were equilibrated with 20 mmol/L phosphate buffer (pH 7.4) containing 0.05 mol/L NaCl. Non-retained lipoproteins (HDL) were collected in the same buffer and retained lipoproteins (LDL) were eluted with 20 mmol phosphate buffer (pH 7.4) containing 0.2 mol/L NaCl. The fractions were dialysed three times against 20 mmol/L phosphate buffer (pH 7.4) containing 0.05 mol/L NaCl and 0.02% sodium azide for 6 h at 4° C.

Extraction of liver lipids

Liver samples were homogenized in 4 volumes of 0.9% NaCl solution. Total lipids in liver homogenates were extracted with chloroform–methanol (2:1) (Folch *et al*., 1957), concentrated by dryness, and dissolved in isopropanol.

Other methods

The concentrations of TC and non-esterified fatty acid (NEFA) in serum or lipoprotein fractions, and the concentration of TG in liver extract were measured using kits (Wako Pure Chemicals, Osaka, Japan). The apoB-100 concentration was determined by single radial immunodiffusion (SRID) assay (Metabolic Ecosystem, Miyagi, Japan). Data are expressed as mean $\pm$ SD. The significance of differences between data was evaluated by Student's paired *t*-test. A level of $p < 0.05$ was regard as significant. For the comparison of methods for serum LDL-C by the N-geneous method with the UC-HS method, linear regression plot and Bland–Altman difference plot (Bland and Altman, 1986) were used.

RESULTS

The serum LDL-C determined by the N-geneous method in serum obtained from heifers was dependent upon the serum volume. Linearity was obtained with up to 20μl of serum in the reaction mixture (data not shown). Based on these data a sample volume of 10μl was used in this study. The intra-assay coefficient of variations from ten measurements of bovine serum LDL-C (12.4 mg/dl) was 4.2%, and the inter-assay coefficient of variations was 7.6%.

The relationship between the values of LDL-C determined by the UC-HS method and those by the N-geneous method is shown in Figure 1A. The regression equation obtained was $y = 0.85x + 2.32$ and the r value was $r = 0.934$ ($p < 0.001$). The Bland– Altman difference plot (N-geneous method $-$ UC-HS method) for paired mean of the two methods is shown in Figure 1B. The mean difference between the measured values for these methods was 0.6 mg/dl with SD of 1.4.

Figure 2 shows the changes in TC, apoB-100 and LDL-C in sera from 12 dairy cows from 4 weeks before parturition to 10 weeks after parturition. The concentration of LDL-C was 11 ± 0.53 mg/dl at 4 weeks before parturition. The values were significantly decreased at parturition, but recovered 4 weeks later and further increased to 17 ± 0.53 mg/dl 10 weeks later. The changes of LDL-C values coincided well with those of apoB-100. The concentration of TC was elevated after parturition, but the extent of changes was small before parturition, in compared with those of apoB-100 and LDL-C.

To investigate the change of LDL-C in fatty liver cows, 4 non-lactating Holstein cows were fasted for 5 days. The concentration of NEFA in serum was elevated significantly from day 1 to day 5 (data not shown). The TG contents in livers were 1.5 ± 0.6 mg/g wet weight at day -10, and 107.2 ± 37.8 mg/wet g at day 4. ApoB-100 and LDL-C in serum decreased significantly from day 4 to day 6, and from day 5 to day 7, respectively, compared with

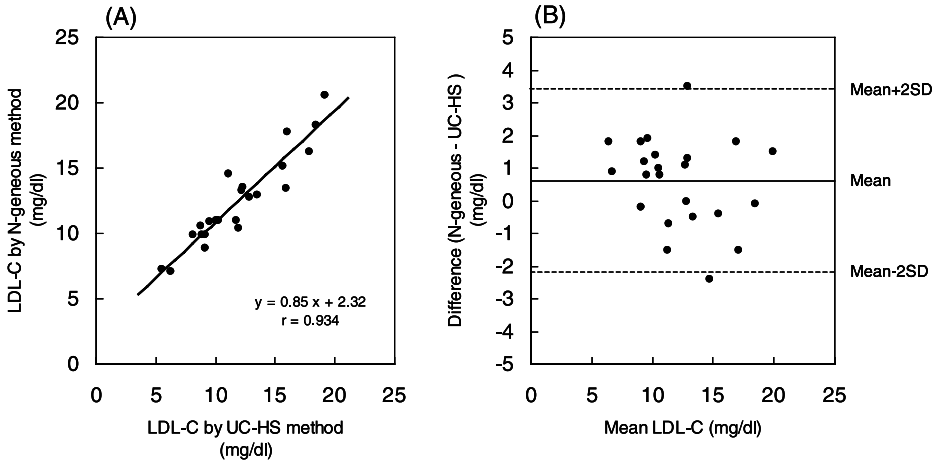

Figure 1. Linear regression plot (A) and Bland–Altman difference plot (B) for the comparison of analyses of serum LDL-C by the N-geneous method all by the UC-HS method. Sera from 3 Holstein male calves, 8 Holstein heifers and 12 lactating cows were used

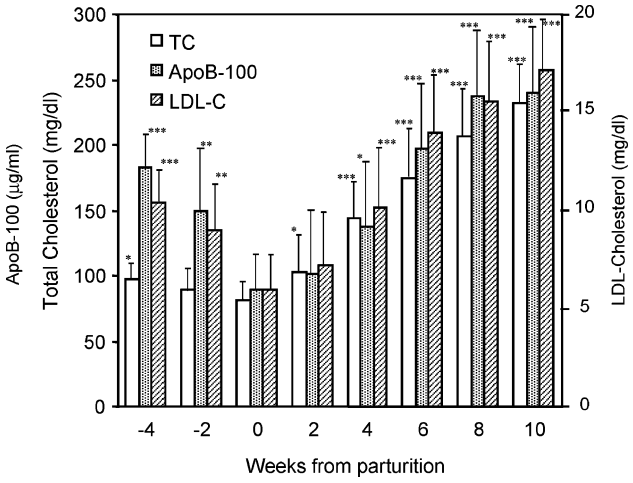

Figure 2. Changes of ApoB-100, total cholesterol and LDL-C in serum (mean ± SD) from 10 dairy cows around parturition. LDL-C was determined by the N-geneous method. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to the respective values at 0 week from parturition (week 0)

those at day 0 (Figure 3). The concentration of TC in serum did not change during fasting, but was significantly decreased at day 7.

A plot of the individual values of apoB-100 and LDL-C in serum, obtained from cows around parturition and cows with fatty liver induced by fasting, is shown in Figure 4. There was a high correlation ($r = 0.876$, $p < 0.001$) between the two parameters.

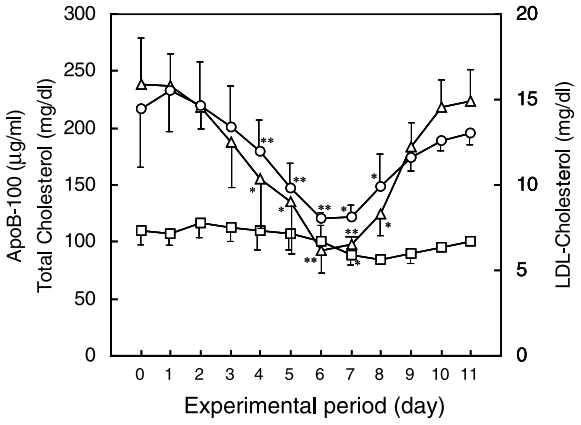

Figure 3. Changes of ApoB-100 (△), total cholesterol (□) and LDL-C (○) in serum (mean ± SD) obtained from experimental fatty-liver cows. Four non-lactating cows was fasted for 5 days and re-fed for 7 days. LDL-C was determined by the N-geneous method. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the respective values at day 0

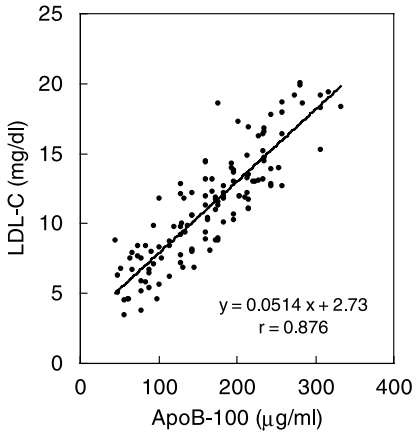

Figure 4. A plot of the individual values of apoB-100 and LDL-C in serum, obtained from cows around parturition and cows with fatty liver induced by fasting. LDL-C was determined by the N-geneous method

DISCUSSION

The N-geneous method is enzymatic method for determination of LDL-C in human serum. In the present study, we investigate whether this method is available for bovine serum, comparing it with the UC-HS method, which is the only quantitative method for LDL-C in bovine serum. Although values from the N-geneous method tended to be higher than those of UC-HS method, the mean difference was small enough (0.6 ± 1.4 mg/dl, Figure 1B). There

was a high correlation between the values from two methods ($r = 0.934$, $p < 0.001$, Figure 1A). These data suggest that the N-geneous method is applicable for the determination of LDL-C in bovine serum. The method is very simple and its reproducibility is acceptable for clinical use in cattle.

It is well known that the concentration of apoB-100 decreases in cows with fatty liver, and measurement of serum apoB-100 is available for the diagnosis of fatty liver and related diseases (Marcos *et al*., 1990a; Oikawa *et al*., 1995; Oikawa and Katoh, 1997, 2002). A decrease of apoB-100 also occurs in cows during the early lactation stage (Marcos *et al*., 1990b; Yamamoto *et al*., 1995), which is probably caused by hepatic TG accumulation. In this study, significant depletion of LDL-C was observed in cows at parturition and with fatty liver induced by fasting, in addition to that of apoB-100 (Figures 2 and 3). The changes of LDL-C were similar to those of apoB-100, and the values of LDL-C were highly correlated with those of apoB-100 (Figure 4). The depletion of serum apoB-100 in cows at parturition and cows with fatty liver might be attributed to the decreased synthesis or secretion of VLDL by the liver (Yamamoto *et al*., 1995). In bovine serum, the activity of cholesteryl ester transfer from HDL to LDL is low compared with the human (Ha and Barter, 1982). Therefore, it is considered that the serum concentration of LDL-C reflects the level of secretion of apoB-100 containing lipoproteins from liver.

For determination of the apoB-100 concentration, SRID assay (Marcosh *et al*., 1989) and enzyme-linked immunosorbent assay (Yamamoto *et al*., 1995) have been reported. However, these methods require antibodies against bovine apoB-100, and are time-consuming. In contrast, the N-geneous method is a simple and rapid; therefore, LDL-C determination by this method may be substituted for apoB-100 determination.

Decreased concentrations of progesterone and glucocorticoid were observed in cows with fatty liver and were thought to be one of the causes of infertility or immune dysfunction (Morrow *et al*., 1979; Watson and Williams, 1987). This is presumably due to an insufficient supply of cholesterol to steroidogenenic organs such as the corpus luteum or adrenal cortex. It is well established that the most important role of LDL is delivery of cholesterol to peripheral tissues via LDL receptors. Rudling and Peterson (1985) clearly demonstrated that the tissue concentrations of LDLs receptors are highest in corpus luteum or adrenal cortex, and that plasma LDLs are closely related to tissue LDL receptors. On the other hand, it was reported that there was no difference in the ability of LDL-C and HDL-C to stimulate progesterone production by cultured bovine corpus luteum cells (Carrol *et al*., 1992). Although the clinical importance of bovine serum LDL-C for steroidogenesis remains unclear, the N-geneous method might to be a useful tool to elucidate this point.

REFERENCES

Bauchart, D., 1993. Lipid absorption and transport in ruminants. *Journal of Dairy Science*, **76**, 3864–3881

Bauchart, D., Durand, D., Laplaud, P.M., Forgez, P., Goulinet, S. and Chapman M.J., 1989. Plasma lipoproteins and apolipoproteins in the preruminant calf, *Bos* spp: density distribution, physicochemical properties, and the *in vivo* evaluation of the contribution of the liver to lipoprotein homeostasis. *Journal of Lipid Research*, **30**, 1499–1514

Bland, J.M. and Altman D.G., 1986. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet*, **1**, 307–310

Burstein, M. and Scholnick, H.R., 1973. Lipoprotein–polyanion–metal interactions. *Advances in Lipid Research*, **11**, 67–108

Carrol, D.J., Grummer, R.R. and Mao, F.C., 1992. Progesterone production by cultured luteal cells in the presence of bovine low- and high-density lipoproteins purified by heparin affinity chromatography. *Journal of Animal Science*, **70**, 2516–2526

Cordle, S.R., Clegg, R.A. and Yeaman, S.J., 1985. Purification and characterization of bovine lipoproteins: resolution of high density and low density lipoproteins using heparin-Sepharose chromatography. *Journal of Lipid Research*, **26**, 721–725

Folch, J., Lees, M. and Sloane Stanley, G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry*, **226**, 497–509

Friedewald, W.T., Levy, R.I. and Fredrickson, D.S., 1972. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clinical Chemistry*, **18**, 499–502

Ha, Y.C. and Barter, P.J., 1982. Differences in plasma cholesteryl ester transfer activity in sixteen vertebrate species. *Comparative Biochemistry and Physiology B*, **71**, 265–269

Hatch, F.T. and Lees, R.S., 1968. Practical methods for plasma lipoprotein analysis. *Advances in Lipid Research*, **6**, 1–68

Marcos, E., Mazur, A., Cardot, P. and Rayssiguier, Y., 1989. Quantitative determination of apolipoprotein B in bovine serum by radial immunodiffusion. *Comparative Biochemistry and Physiology B*, **94**, 171–173

Marcos, E., Mazur, A., Cardot, P. and Rayssiguier, Y., 1990a. Serum apolipoprotein B and A-I and naturally occurring fatty liver in dairy cows. *Lipids*, **25**, 575–577

Marcos, E., Mazur, A., Cardot, P. and Rayssiguier, Y., 1990b. The effect of pregnancy and lactation on serum lipid and apolipoprotein B and A-I. *Journal of Animal Physiology and Animal Nutrition*, **64**, 133–138

Morrow , D.A., Hillman, D., Dade, A.W. and Kitchen, H., 1979. Clinical investigation of a dairy herd with the fat cow syndrome. *Journal of the American Veterinary Medical Association*, **174**, 161–167

Oikawa, S. and Katoh, N., 1997. Reduced concentrations of apolipoproteins B-100 and A-I in serum from cows with retained placenta. *Canadian Journal of Veterinary Research*, **61**, 312–314

Oikawa, S. and Katoh, N., 2002. Decreases in serum apolipoprotein B-100 and A-I concentrations in cows with milk fever and downer cows. *Canadian Journal of Veterinary Reseach*, **66**, 31–34

Oikawa, S., Katoh, N., Kawawa. F. and Ono, Y., 1995. Decreased serum apolipoprotein B-100 and A-I concentrations in cows with ketosis and left displacement of the abomasum. *American Journal of Veterinary Research*, **58**, 121–125

Rifai, N., Iannotti, E., DeAngelis, K. and Terence, L., 1998. Analytical and clinical performance of a homogeneous enzymatic LDL-cholesterol assay compared with the ultracentrifugation–dextran sulfate–Mg^{2+} method. *Clinical Chemistry*, **44**, 1242–1250

Rudling, M.J. and Peterson, C.O., 1985. A simple binding assay for the determination of low-density lipoprotein receptors in cell homogenates. *Biochimica et Biophysica Acta*, **833**, 359–365

Watson, E.D. and Williams, L.A., 1987. Influence of liver fat on postpartum hormone profiles in dairy cows. *Animal Production*, **45**, 9–14

Yamamoto, O., Oikawa, S. and Katoh, N., 1995. Enzyme-linked immunosorbent assay for serum apolipoprotein B-100, a major triglyceride-transport protein in dairy cows. *American Journal of Veterinary Research*, **56**, 1413–1417

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