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Fluorometric Method for Measuring Plasma Tartrate-Resistant Acid Phosphatase Isoform 5b and Its Application in Cattle

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ABSTRACT. This study determined the appropriate biochemical assay for measuring plasma tartrate-resistant acid phosphatase isoform 5b (TRAP5b) activity; this information is important to clarify the relationship between plasma TRAP5b and known biochemical bone markers in cattle. When plasma TRAP5b was measured using fluorometric and spectrophotometric methods, hemolysis products in plasma did not affect the former method. In plasma from healthy cattle, there was a good correlation (r=0.66) between the 2 methods. In age-related profiles, plasma TRAP5b (r=–0.53), hydroxyproline (HYP, r=–0.56) and bone-specific alkaline phosphatase (BALP, r=–0.44) showed significant negative correlations with age; these three parameters decreased until 4 or 5 years of age and then remained constant. There were significant correlations between TRAP5b and HYP (r=0.83) or BALP (r=0.83). Our results show that the fluorometric assay can be performed with a high degree of precision and reproducibility without interference from hemolysis, and that the age-related changes in plasma TRAP5b, HYP, and BALP constitute additional background values for clinical guidance in bovine medicine.

KEY WORDS: biochemical bone marker, cattle, fluorometry, plasma, tartrate-resistant acid phosphatase isoform 5b (TRAP5b).

Bone remodelling, consisting of alternating bone growth and turnover, is the main process of bone metabolism [24]. Information on bone metabolism can be determined by measuring biochemical bone markers in blood and urine derived from the activity of the cells involved [1, 14, 23]. In veterinary medicine, especially in canine and equine medicine, bone markers are used as non-invasive diagnostic tools for monitoring age-related trends in bone formation and resorption, the skeletal effects of exercise, fracture healing, skeletal neoplasia, and subchondral bone changes associated with osteoarthritis [1, 14]. Similarly, because skeletal disease (e.g., bone fracture, osteoarthritis, and osteomalacia) is often encountered in bovine practice, the assessment of bone markers may be helpful for determining diagnosis and prognosis. However, knowledge of bone markers is still limited to adult dairy cows during pregnancy and lactation [16, 17, 19, 31–33], cows with experimental hypocalcaemia [14], and cows fed a diet to prevent milk fever [7, 11, 18, 24].

Tartrate-resistant acid phosphatase (TRAP) belongs to a family of acid phosphatase isoenzymes found in bone, erythrocytes, platelets, the spleen, and the prostate [6]. TRAP in plasma or serum can be classified into three types: TRAP isoform 5a (TRAP5a), TRAP isoform 5b (TRAP5b), and non-type 5 TRAP [10]. The osteoclast-specific isoform TRAP5b is a lysosomal enzyme secreted by activated osteoclasts, whereas TRAP5a and non-type 5 TRAP are thought to be derived from activated macrophages and from erythrocytes and platelets, respectively [6, 10, 22]. Hence, it is necessary to measure only TRAP5b as a plasma marker for osteoclasts.

A practical spectrophotometric assay for TRAP5b in bovine blood specimens that uses para-nitrophenylphosphate (pNPP) as the substrate has been described [18, 24, 31–33]. In this method, heparin was added as a specific inhibitor of TRAP5a activity, and the optimum pH was thought to be 6.6 [20, 21, 33]. However, this method has low specificity for hydrolysed bone, because non-type 5 TRAP is released from erythrocytes and platelets by mechanical lysis during blood collection and clotting [20] and consequently interferes with the end-products of pNPP hydrolysis spectrophotometrically [10, 20]. Hemolysis often occurs during blood collection in bovine veterinary practice; we sought to determine an appropriate biochemical assay for measuring plasma TRAP5b activity that was not affected by hemolysis. A recent report on humans found that a fluorometric method using naphthol-ASBI-phosphate (N-ASBI-P) as the substrate could be used to measure plasma TRAP5b activity without being affected by hydrolysis [10]. Here, we compared these fluorometric and spectrophotometric methods to determine a hypothesis that the former method has better analytical precision than another for measuring TRAP5b activity in bovine blood specimens. In addition, we clarified the relationship between plasma TRAP5b and known biochemical bone markers, such as hydroxyproline (HYP) and bone-specific alkaline phosphatase (BALP), in healthy cattle at various ages.
MATERIALS AND METHODS

Animals and study design: First, to determine the appropriate biochemical assay for plasma TRAP5b, heparinized blood samples were collected from six healthy male Jersey calves (age 2 to 3 weeks old) kept in the Veterinary Teaching Hospital, Iwate University, for a veterinary education programme. The blood was withdrawn from the jugular vein of each calf into two 6-mL heparinized blood collection tubes (BD Vacutainer LH102 IU Plus Blood Collection Tubes, BD, Franklin Lakes, NJ, U.S.A.) with an 18-G × 3.8-mm blood collection needle (Nipro Medical, Osaka, Japan). Parallel analyses using the fluorometric and spectrophotometric methods were conducted using plasma from each calf prepared in two different ways. Half of the blood was centrifuged immediately to obtain plasma without hemolysis and the remaining half was separated by centrifuging the blood after inducing hemolysis mechanically (plasma with hemolysis) by rapidly aspirating and ejecting the blood through a 21-G × 3.8-mm needle (Flomax hypodermic needle, Nipro Medical) attached to a 5-mL disposable syringe (Nipro Medical) 5 times, and then repeating the process five more times with a 20-G × 7.0-mm needle (Terumo hypodermic needle, Terumo Medical, Tokyo, Japan) attached to the same syringe. Hemoglobin concentrations in plasma were measured by a pocH-100iV Diff (Sysmex Corporation, Kobe, Japan). All specimens were frozen at −50°C until TRAP5b activity was analysed.

To determine the age-related changes in bone markers, plasma TRAP5b, HYP, and BALP levels were assessed in healthy Japanese Black cattle (15 males, 50 females; age, 15 days to 11.4 years) kept at the research farm of the Field Science Centre, Iwate University. All animals were declared healthy after clinical examinations. Heifers and cows within 3 weeks of parturition were excluded. The animals were fed grass hay and commercial grains, and were given water ad libitum, except for nine calves aged less than 2 months (4 males, 5 females) that could access their mothers to drink milk. All blood samples were withdrawn from the jugular vein into 6-mL heparinised blood collection tubes through a 21-G × 3.8-mm blood collection needle (Nipro Medical) between 10:00 and 12:00 hr on the same day. The blood was centrifuged immediately to separate plasma, which was then frozen at −50°C until analysed for TRAP5b, HYP, and BALP.

The study protocol and experimental design were approved by the Iwate University Laboratory Animal Care and Use Committee.

Biochemical assays for plasma TRAP5b: In the fluorometric assay, plasma TRAP5b activity was measured using the method of Jancikla and others [10], with slight modification. Briefly, 10 μL of the plasma sample were added to 50 μL of substrate consisting of 2.5 mM N-ASBI-P (Wako Pure Chemical Industries, Osaka, Japan) in 100 mM sodium acetate buffer containing 50 mM sodium tartrate (Wako Pure Chemical Industries), 2% Nonidet P-40 (NP-40, BioVision, Mountain View, CA, U.S.A.), 1% ethylene glycol monomethyl ether (EGME, Wako Pure Chemical Industries), and heparin (23 U/mL) adjusted to pH 6.6. The required amount of N-ASBI-P was dissolved in EGME and NP-40 before adding the aqueous buffer. The reaction was carried out for 30 min at 37°C, and quenched by adding 125 μL of 0.1 M NaOH containing 0.05% NP-40. Reagent blanks were prepared for each plasma sample by adding 125 μL of 0.1 M NaOH containing 0.05% NP-40. A standard calibration curve was constructed using acid phosphatase solutions of known concentration (Wako Pure Chemical Industries). Fluorescence was measured using an ARVO MX/Light 1420 Multilabel Counter (Perkin-Elmer, Waltham, MA, U.S.A.) with an excitation wavelength of 405 nm and a peak emission wavelength of 535 nm. Under these measurement conditions, the intra- and interassay coefficients of variance (CVs) were 10.6% (n=20) and 15.0% (n=5), respectively.

In the spectrophotometric assay, plasma TRAP5b activity was measured using the method of Lau and others [13], with some modifications [20, 21, 33]. Briefly, plasma was diluted five-fold in distilled water, and incubated for 1 hr at 37°C. Then, 50 μL of the diluted plasma sample were added to 50 μL of substrate consisting of 100 mM pNPP (Wako Pure Chemical Industries) in 200 mM sodium citrate buffer containing 80 mM sodium tartrate (Wako Pure Chemical Industries), 200 mM sodium chloride (Kanto Chemical, Tokyo, Japan), and heparin (42 U/mL, Kanto Chemical) adjusted to pH 6.6. The reaction was carried out for 1 hr at 37°C, and quenched by adding 50 μL of 1 M NaOH. A standard calibration curve was constructed using para-nitrophenol solutions of known concentration (Kanto Chemical). The absorbance was measured at 405 nm in an ARVO MX/Light 1420 Multilabel Counter. The intra- and interassay CVs were 6.3% (n=20) and 10.9% (n=5), respectively.

Biochemical assays for plasma HYP and BALP: Plasma HYP was measured spectrophotometrically using the method of Dabevo and Struck [5]. Plasma BALP was measured using a spectrophotometric method [3, 30].

Statistical analysis: All numerical data are expressed as means ± standard deviation (SD). In the hemolysis experiment, one-way repeated measures analysis of variance (ANOVA), followed by Dunnett’s test, were performed to detect significant differences in TRAP5b activity compared to activity in plasma without hemolysis measured using the fluorometric method. With the data for the 65 Japanese Black cattle, Pearson’s correlation test was performed to evaluate the correlation between plasma TRAP5b activities measured using the fluorometric and spectrophotometric methods. Next, these 65 cattle were assigned to 5 groups: 15 males aged 15 days to 11.8 months (mean: 5.3 months old; bull-calf group); 13 female calves aged 12 days to 10 months (3.5 months old; female-calf group); 13 heifers aged 12.4 to 23.1 months (16.1 months old; heifer group); 17 cows aged 2.1 to 4.3 years (3.0 years old; younger-cow group); and 7 cows aged 5.4 to 11.4 years (7.8 years old; older-cow group). Differences in plasma bone markers between the bull-calf and female-calf groups were compared using Student’s t-test to evaluate the gender difference.
at the same age. The differences in plasma bone markers among the 4 groups of females (i.e., the female-calf, heifer, younger-cow, and older-cow groups) were compared using one-way ANOVA and the Tukey-Kramer multiple comparison test. Pearson’s correlation test was used to evaluate the significance of the correlations between age and each bone marker, and between TRAP5b and HYP or BALP. The threshold for statistical significance was \( P<0.05 \).

RESULTS

Figure 1 shows the TRAP5b activity in plasma with or without hemolysis measured using the fluorometric and spectrophotometric methods. Hemoglobin concentrations in plasma with hemolysis were between 0.1 and 0.5 g/dl (n=6). Hemolysis did not interfere with plasma TRAP5b activity measured using the fluorometric method, whereas TRAP5b activity was significantly higher (\( P<0.01 \)) in plasma with hemolysis than that without hemolysis when measured using the spectrophotometric method. No significant difference in TRAP5b activity in plasma without hemolysis was observed between the two methods, although there was a great deal of variation with the spectrophotometric method.

Figure 2 shows the relationship between plasma TRAP5b activities measured using the fluorometric and spectrophotometric methods in 65 healthy Japanese Black cattle. There was a significant positive correlation between the two methods (\( r=0.66, P<0.01 \)).

Figure 3 shows the mean (SD) plasma concentrations of the biochemical bone markers in the 65 cattle. There was no significant difference in any bone marker between the bull-calf and female-calf groups. Within the four groups of females, the ranking of plasma TRAP5b activity was as follows: female-calf group > heifer group > younger-cow group ≥ older-cow group. The age-related changes in plasma HYP and BALP resembled those for plasma TRAP5b (\( P<0.01 \)). For females older than 1 year, the heifer group had significantly higher plasma TRAP5b and HYP activities than the older-cow group (\( P<0.01 \)).

Figure 4 plots the plasma concentrations of each bone marker as a function of age for the 65 cattle. Plasma TRAP5b (\( r=-0.53 \)), HYP (\( r=-0.56 \)), and BALP (\( r=-0.44 \)) all showed significant negative correlations with age (\( P<0.01 \)), which decreased until 4 or 5 years of age and remained constant thereafter.

Figure 5 shows scatter plots of the relationship between plasma TRAP5b and HYP or BALP. Plasma TRAP5b showed significant positive correlations with plasma HYP (\( r=0.83, P<0.01 \)) and BALP (\( r=0.83, P<0.01 \)).

DISCUSSION

Bone markers in the blood and urine of cows include TRAP5b, HYP, pyridinoline, deoxypyridinoline, and the carboxy-terminal telopeptide of type 1 collagen as bone resorption indices, and BALP and osteocalcin as bone formation parameters [1]. It is difficult to quantify the precise urine volume of cattle, although many of these indicators are evaluated using urinary concentrations [1]. Therefore, the assessment of blood bone markers as a routine laboratory procedure should prove feasible in bovine practice. Here, we found that TRAP5b activity in plasma with hemolysis was significantly higher when measured using the spectrophotometric method than TRAP5b activity in plasma with-
Table 1. Plasma bone markers in 65 cattle at various ages. TRAP5b (tartrate-resistant acid phosphatase isoform 5b) was measured by the fluorometric method. HYP, hydroxyproline. BALP, bone specific alkaline phosphatase. Significant difference between two groups of females (\(* P<0.05, ** P<0.01\)).

Fig. 3. Plasma bone markers in 65 cattle at various ages. TRAP5b (tartrate-resistant acid phosphatase isoform 5b) was measured by the fluorometric method. HYP, hydroxyproline. BALP, bone specific alkaline phosphatase. Significant difference between two groups of females (\(* P<0.05, ** P<0.01\)).

out hemolysis measured using the fluorometric method. We selected a fluorometric method for the present investigation, because TRAP isolated from the bovine bone matrix resembled that originating from the human osteoclasts from the viewpoint of biological characteristics [12]. Moreover, bovine TRAP was utilized as a standard substance for an assay of human TRAP5b activities [13] and for a kinetic assay of TRAP5b in human serum [20].

In plasma specimens from 65 healthy Japanese Black cattle, TRAP5b measured by the fluorometric method was positively correlated with that measured by the spectrophotometric method, although activities in the former was somewhat lower than those in the latter (Fig. 2). Under our study conditions, because the blood was sampled through a 21-G needle which was narrower than that (18-G) usually used in bovine practice, hemolysis may occur easily. These data suggest that the fluorometric method eliminates interference caused by non-type 5 TRAP.

HYP, an amino acid contributing to collagen orientation within the bone matrix, is used as a marker of bone resorption in cattle [24, 33] and horses [3, 25]. Sato et al. [33] reported that serum HYP concentrations were positively correlated with serum TRAP5b activities measured using the spectrophotometric method in dairy cows (\(r=0.64\)), suggesting that both markers similarly reflected the bone resorption in cattle. This finding was in agreement with our data (\(r=0.65\), data not shown) obtained from the spectrophotometric method in 65 healthy Japanese Black cattle. Interestingly, a relatively high correlation coefficient (\(r=0.83\)) between plasma HYP and plasma TRAP5b using the fluorometric method was noted. This demonstrates that the fluorometric method possesses a high degree of precision compared to the spectrophotometric method for measuring bovine serum TRAP5b activities.

The use of HYP is controversial, because plasma HYP is influenced by the diet and the metabolism of non-bony collagen, such as that in muscle, skin, and liver [14]. In contrast, it has been reported that TRAP5b is well correlated with the number of osteoclasts and serves as a specific marker of osteoblast activity and bone resorption [4]. Therefore, plasma TRAP5b appears to be a useful, specific alternative to plasma HYP for evaluating bone resorption in cattle.

To our best knowledge, there is little information on the age-related patterns of plasma bone markers in cattle, although there have been many reports in dogs and horses [2, 26]. In our study, two bone resorption markers (TRAP5b and HYP) and one bone formation marker (BALP) were higher in younger animals and decreased until 4 or 5 years of age, and remained constant thereafter. These findings imply that bone resorption and formation are balanced during remodelling in skeletally mature cattle, comparable to reports on dogs [1] and young subjects [29]. Allen [1] described the process of bone turnover as occurring through two fundamentally different processes: modelling and remodelling. In modelling, bone formation and bone resorption are spatially and temporally independent of one another, whereas remodelling involves the sequential removal and replacement of bone at discrete sites by the concerted actions of the osteoclasts and osteoblasts that comprise the bone multicellular units.

BALP, a non-collagenous protein secreted by osteoblasts, is essential for bone mineralisation, and is a highly specific marker of osteoblast function [9]. In our study, plasma BALP showed a significant positive correlation with plasma TRAP5b (\(r=0.83\)), suggesting that the activities of osteoclasts and osteoblasts were balanced during the process of bone turnover in our cattle. Because the bone formation markers including BALP have been reported to be closely correlated with the bone resorption markers like TRAP5b in clinically healthy humans [34] or horses [26], these two bone processes are believed to be couple in physiological states. Under certain diseased conditions, however, the dissociation between bone formation and resorption was seen [34, 35]. For example, the parturition is believed to represent physiological situations of the calcium stress, and thereby contributes to drastic changes in calcium homeostasis. The calcium homeostasis of parturient cows depends on intestinal calcium absorption, and the bone resorption is suppressed or delayed for a week or more [27, 28]. Physiological changes in plasma TRAP5b as well as bone markers such as HYP and BALP around parturition are necessary to examine in further studies, although there were several reports dealing with the spectrophotometric method for measuring plasma TRAP5b in cows [11, 32, 33].

Gender differences in blood bone markers have been reported in adult humans and baboons [8, 9]. Because our data were collected from males before puberty (younger
than 1 year old), further studies are necessary to examine the gender-related changes in adult cattle.

In conclusion, our data suggest that the fluorometric TRAP5b assay using N-ASBI-P as the substrate can be performed with a high degree of precision and reproducibility without interference from hemolysis. This assay method can be executed more rapidly than the spectrophotometric method, where a fluorescence meter is employed. Finally, the data of the present study also suggest that the age-related changes in plasma TRAP5b in conjunction with HYP and BALP constitute additional background values for clinical guidance in bovine medicine.

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