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Detection of methionine- and alanine-recombinant bovine somatotropins and their induced antibodies in serum and milk of cows suggests blood-milk barrier specificity for these compounds

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ABSTRACT

The elimination of recombinant bovine somatotropin (rbST) and its induced antibodies through milk of 2 formulations is studied to propose a control strategy for its use or abuse. Two dairy cows were treated with alanine-rbST (Ala-rbST), which is identical to endogenous bovine somatotropin, and ten dairy cows were treated with methionine-rbST (Met-rbST), which differs by 1 amino acid from endogenous bovine somatotropin. We developed a liquid chromatography–tandem mass spectrometry (LC-MS/MS) method able to measure rbST at a decision limit (CCα) of 0.8 ng/mL and 2.3 ng/mL for serum and milk, respectively. The results show that the administered Ala-rbST is transferred from blood to milk but that this is not the case for Met-rbST. This suggests a blood-milk barrier-related specificity for these compounds. In addition, rbST-induced antibodies were formed in animals treated with Ala-rbST and those treated with Met-rbST. In both treatments, the rbST-induced antibodies were transferred from blood to milk, showing no blood-milk barrier specificity for these antibodies. These elimination patterns show that, for enforcement purposes, the detection of rbST-induced antibodies in tank milk can serve to screen for rbST administration, and subsequent confirmatory serum analysis by LC-MS/MS is needed to identify whether Ala-rbST or Met-rbST has been used.
Key words: recombinant bovine somatotropin, blood-milk barrier, dairy cow, biomarker, bST

INTRODUCTION

Recombinant bovine somatotropin (**rbST**) is a 191 amino acid–long protein hormone, commercially available in slow-release formula injection preparations in 2 forms: (1) identical to endogenous bovine somatotropin (**bST**) with alanine as N-terminal amino acid (**Ala-rbST**), and (2) 1 amino acid difference from endogenous bST, the N-terminal alanine replaced by methionine (**Met-rbST**). Recombinant bST is administered to dairy cows to enhance milk production, which can then increase up to 25% (Butler, 1999). Normally, 500 mg of rbST subcutaneously injected in a slow-release formula allows a low administration frequency of just once every 2 wk. According to the size of rbST (22 kDa), most subcutaneously injected rbST is expected to enter the central lymph system by the lymph absorption pathway. From there, rbST will subsequently migrate into blood circulation (McLennan et al., 2005). Next to the presence of the rbST in blood circulation, specific antibodies against rbST (anti-rbST) can be formed due to rbST treatment, which will be referred to as "anti-rbST antibody-type biomarker" in the following text, according to its definition by Califf (2018). It is expected that this occurs even when the rbST amino acid sequence is identical to that of endogenous bST, similar to humans treated with human somatotropin (Binder et al., 2019). This immune response then is due to rbST aggregate formation during production, shipping, and storage of rbST in formulas. In general, protein aggregates of multiple monomers result in a repetition of monomer parts, which are associated with pathogenic patterns and therefore trigger an immune response (Rosenberg, 2006; Moussa et al., 2016; Nabhan et al., 2020).

The use of rbST is forbidden in the European Union according to Council Decision 1999/879/EC, and control of its misuse is required (European Council, 1999). For control purposes, the presence of either rbST or anti-rbST antibody-type biomarker in the cow can be used. Because blood sampling is not as easy as taking tank milk samples, and moreover for regulatory simplicity, tank milk is preferred (for example, inspectors in the Netherlands are not allowed to draw blood). Therefore, the present study was undertaken to establish whether

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both rbST and the anti-rbST antibody-type biomarker transfer from blood to milk.

Because the method to determine these compounds in blood (Smits et al., 2015) did not work for milk, the method first had to be adjusted. After subcutaneous administration, rbST itself and anti-rbST antibody-type biomarker are expected to be present in the bloodstream. However, transfer from blood to milk is a selective process and requires active cellular transport, as the blood-milk barrier is highly impermeable to prevent diffusion of blood and milk components and to protect the calf after birth (Wall et al., 2015). Only specific substances are transported from blood to milk, for instance, by transcytosis, 1 route among 5 major known routes (Shennan and Peaker, 2000). For our proteins of interest, rbST and the anti-rbST antibody-type biomarker, excretion into the milk is expected because endogenous bST is a bioactive compound that regulates the growth of newborn calves via their mother's milk (Grosvenor et al., 1993). In addition, newborn calves are agammaglobulinemic, which means they have an immature immune system and no detectable immunoglobulins. They are protected by passive immunity supplied by the colostrum and milk from their mothers; therefore it is also expected that the anti-rbST antibody-type biomarker is transferred from the blood to the colostrum and milk via an Fc receptor–mediated mechanism (Hurley and Theil, 2011). Because this mechanism is Fc-based, all IgG found in blood, including rbST-induced antibodies, would thus end up in milk (Mehra et al., 2006; Gapper et al., 2007).

Four liquid chromatography–tandem mass spectrometry (**LC-MS/MS**)-based methods have been described that are able to specifically detect rbST in serum at relevant levels, that is, below 10 ng/mL (Le Breton et al., 2009; Smits et al., 2015; Castigliego et al., 2016; Robert et al., 2017). Results of rbST measurements in incurred milk samples were presented by Welsh et al. (2019), but unfortunately the authors did not describe how rbST in the milk was measured. Until now, rbST has only been detected at high levels in spiked blank milk samples, and rbST data in incurred milk samples is lacking (Le Breton et al., 2010). Regarding the measurement of the anti-rbST antibody-type biomarker, many methods on different platforms have been developed for both serum and milk, such as ELISA (Rochereau-Roulet et al., 2011; Castigliego et al., 2017), Western blotting (Pinel et al., 2005), and flow cytometric immunoassay (**FCIA**; Ludwig et al., 2012; Smits et al., 2012, 2013). In addition, a microarray smartphone readout has been described for the detection of anti-rbST antibody-type biomarkers in milk (Ludwig et al., 2014, 2015). In the present study, the anti-rbST antibody-type biomarker in milk and serum was measured by using the previously developed FCIA method (Smits et al., 2012; Ludwig et al., 2012). Eventually, measuring both rbST and the anti-rbST antibody-type biomarker in serum and milk of treated cows enabled us to study the transfer of rbST and its antibodies from serum to milk and to propose a screening strategy for rbST use or abuse based on anti-rbST antibody-type biomarkers in milk.

MATERIALS AND METHODS

Chemicals

The rbST standard was obtained from the National Hormone and Peptide Program (Torrance, CA). We obtained rbST, Posilac sometribove zinc suspension for injection (Met-rbST), and Excipient (sesame oil and aluminum monostearate) for injection from Elanco (Indianapolis, IN). The rbST for injection (Ala-rbST, Hilac) was obtained from LG (Seoul, South Korea). An isotopic-labeled bST peptide AFPAMSLSGLFA-NAVLR, with incorporation of $^{13}C_6{}^{15}N_4$-alanine, resulting in a total mass increase of 10 Da, and a synthetic analog of the rbST peptide MFPAMSLSGLFANAVLR were obtained from Bachem (Bubendorf, Switzerland). Pierce bicinchoninic acid protein assay and the Finnpipette Novus multichannel electronic and monolithic microcolumns disposable automation research tips protein A were purchased from Thermo Fisher Scientific (Rockford, IL); ammonium sulfate, hydrochloric acid, potassium phosphate, sodium hydroxide, sodium phosphate, and the ultrasonic cleaner from VWR International (Amsterdam, the Netherlands); trypsin, tris(hydroxymethyl)aminomethane, iodoacetamide, dimethyl sulfoxide, and DL-dithiothreitol from Sigma-Aldrich Chemie (Zwijndrecht, the Netherlands); methanol and acetonitrile from Biosolve (Valkenswaard, the Netherlands); and formic acid from Actu-All chemicals (Oss, the Netherlands). Protein LoBind Tubes (1.5 and 2.0 mL), Protein LoBind 96 deep-well plates (1.0 and 2.0 mL), and a table centrifuge, model 5810R, were purchased from Eppendorf (Hamburg, Germany); a GR 20-22 ultracentrifuge from Jouan (Saint-Herblain, France); Snijder test tube rotator from Omnilabo International (Breda, the Netherlands); and Zymark TurboVap from Biotage (Upsala, Sweden). Bond Elut Plexa 30-mg solid-phase extraction columns were purchased from Agilent Technologies (Amstelveen, the Netherlands).

Serum and Milk Samples

To study rbST elimination kinetics via milk, both serum and milk samples were collected from 2 controlled animal experiments with nonpregnant lactating Hol-

stein Frisians cows (varying in stages of lactation and aged 2–7 yr; information per individual cow is shown in Supplemental Table S1, https://doi.org/10.3168/jds.2020-19209). Cows were housed indoor as a group, to enable sight, noise, and smell contact, but physically separated. Cows were fed roughage and concentrate and had ad libitum access to water. The same treatment schedule was used for both animal experiments: an adaptation period of 2 wk was taken into account before starting the treatment; then cows were treated every second week by subcutaneous injections of rbST according to the manufacturers' guidelines. Milk yields per animal were measured by weighing and registered daily. Blood and milk samples were collected approximately 15 min before the injection, as well as both 1 d and 1 wk after each injection. Additionally, following the last (i.e., the fourth and third) subcutaneous injections for experiments 1 and 2, respectively, blood and milk samples were collected daily for 2 wk; this is schematically presented in Figure 1. In the first study, 8 cows were injected with 500 mg of Met-rbST in a slow-release formula, and 2 cows followed the same treatment schedule, but with a similar slow-release formula without rbST (blank controls). In the second study, 2 cows were treated with a new batch of Met-rbST slow-release formula, and 2 additional cows were treated with a 500 mg Ala-rbST slow-release formula. Due to the presence of multiple recent blank materials, from an ethical point of view, we chose not to include a blank cow. The Ala-rbST in this formula is identical to the peptide sequence of endogenous bST. Blood samples were stored at room temperature for 4 h to coagulate. After coagulation, the samples were centrifuged for 10 min at 3,000 × *g*, and serum was collected and stored at −20°C until further analysis. Milk samples were stored at −20°C directly until further analysis. Both studies were authorized by the ethical committee of the Animal Sciences Group of Wageningen University and Research Centre, in Lelystad and Wageningen, the Netherlands, respectively.

Preparation and Purification of Polyclonal Antiserum

Preparation and purification of polyclonal antiserum against rbST was as previously described by Heutmekers et al. (2007).

Pretreatment of Serum and Milk Samples for rbST Analysis

Extraction of rbST from blood and milk samples was based on the rbST extraction protocol for serum developed by Smits et al. (2015), with further improvements being made for blood serum and small adaptations for milk serum samples. Improvements to the serum extraction method comprised addition of Tween 20 during affinity purification, and, for LC-MS/MS measurements, a column with a smaller internal diameter was used for further improvement of the method. In short, serum samples were thawed, and the rbST in the serum samples was extracted and concentrated by affinity binding using polyclonal anti-rbST ammonium sulfate purified antibodies that were immobilized on monolithic microcolumns loaded with protein A (Smits et al., 2015). Protein A–loaded monolithic microcolumns were placed on the Finnpipette, which is an automated device having a repetitive cycling function. First, the monolithic microcolumns were washed 10 times with 150 µL of 10 m*M* PBS with 0.05% Tween-20 (**PBST**), pH 7.4. Adherent solution was removed from the column by air pressure. Next, 75 µL of 0.1 mg/mL of the ammonium sulfate–precipitated polyclonal anti-rbST antibodies, resuspended in 10 m*M* PBST, pH 7.4, was transferred over the column 1,000 times back and forward. After the final cycle, adherent solution was removed from the column by air pressure. To remove

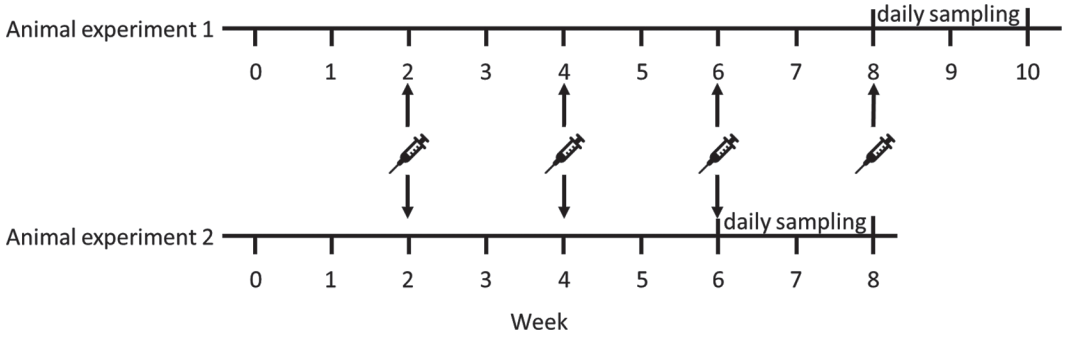

Figure 1. Schematic representation of the treatment schedules of animal experiments 1 and 2. In experiment 1, cows were treated with blank formula (n = 2) and methionine-recombinant bovine somatotropin (Met-rbST) formula (n = 8), and in animal experiment 2 cows were treated with Met-rbST (n = 2) and Ala-rbST (n = 2) formulas. Syringes show the moment of treatment with, respectively, Met-rbST, Ala-rbST, or blank in slow-release formulas.

unbound antibodies, the monolithic microcolumns were washed twice by 10 cycles of 150 μL of 10 m*M* PBST, pH 7.4. These freshly prepared monolith microcolumns immobilized with ammonium sulfate–precipitated polyclonal anti-rbST antibodies were then directly used for rbST enrichment. For immunoenrichment, 1 mL of serum sample, including matrix-matched spike controls, were diluted with 1 mL of 10 m*M* PBST, pH 7.4. The sample was transferred 2,000 times (300 μL per cycle) back and forward over the anti-rbST immobilized monolithic microcolumn. Adherent sample was removed from the column by air pressure. To remove remaining unbound sample, the monolithic microcolumns were washed 10 times with 150 μL of 10 m*M* PBST pH 7.4. Captured rbST was then eluted from the monolith microcolumn by 50 μL of 200 m*M* NaOH (20 μL per cycle, 150 times). The eluate was collected and stored at −20°C until tryptic digestion.

To extract rbST from milk samples, the same steps were used, except that, after thawing, the milk was first centrifuged at 3,000 × *g* for 10 min at 4°C, and 2 mL from the aqueous layer was used and diluted with 2 mL of 10 m*M* PBST. For extraction, the sample was divided into 2 portions, and these portions were consecutively transferred 1,000 times over a single monolith microcolumn. Thereafter, rbST extraction from the milk samples followed the same procedure as that described for serum.

Digestion and Cleanup of the Immunoaffinity-Enriched rbST Serum and Milk Extracts

For digestion of proteins with trypsin, 50-μL stored sample extracts were thawed, and 50 μL of 200 m*M* Tris, pH 8, was added. The pH of the obtained solution was adjusted to between 8 and 8.5 with 5 μL of 5% formic acid. To reduce the formation of sulfur bridges, 5 μL of 45 m*M* DL-dithiothreitol in 200 m*M* Tris, pH 8, was added, and the solution was mixed and incubated for 30 min at 37°C. The solution was cooled to room temperature, and 5 μL of 0.1 *M* iodoacetamide in 200 m*M* Tris, pH 8, was added for methylation of the cysteine residues by mixing on a vortex and incubation for 30 min at room temperature in the dark. For protein digestion, 5 μL of 1 μg/μL freshly prepared trypsin in 200 m*M* Tris, pH 8, was added, followed by addition of 20 μL of acetonitrile, mixing by vortex, and incubation for 1 h at 37°C. Digestion was terminated by addition of 0.9 mL of 5% formic acid, and an internal standard of an isotope-labeled bST peptide was added. This labeled peptide is a replicate of the 17 amino acids of the N-terminal end of endogenous bST (AFPAMSLSGLFANAVLR) and differs only in the N-terminal amino acid by incorporation of $^{13}C_6{}^{15}N_4$-alanine, resulting in a total mass increase of 10 Da. The sample digest was transferred to a Bond Elut solid-phase extraction column previously conditioned with 1 mL of methanol and 1 mL of 5% formic acid in water. Then the column was washed with 1 mL of 15% acetonitrile and eluted with 0.5 mL of water:acetonitrile:formic acid (15:75:10 vol/vol/vol). The eluate was collected in a tube already containing 50 μL of dimethyl sulfoxide and evaporated to approximately 100 μL in the TurboVap at 55°C under 10 psi N_2. After cooling to room temperature and mixing, the sample was ready for LC-MS/MS analysis.

LC-MS/MS Analysis

Analysis was performed on an I-Class ultra-performance liquid chromatography system connected to a Xevo triple quadrupole mass spectrometer (Waters, Manchester, UK), operated in the multiple reaction monitoring mode. Using 20 μL of the final sample extracts, chromatographic separation was performed on an Acquity Peptide CSH C_{18}, 130 Å, 1.7 μm, 1 × 100 mm column (Waters). Mobile phase A consisted of 10% acetonitrile with 0.1% formic acid and 0.01 *M* ammonium formate, and phase B consisted of 90% acetonitrile with 0.1% formic acid and 0.01 *M* ammonium formate. The flow rate was set at 0.175 mL/min, and mobile-phase composition of 80% A and 20% B was used for the first 30 s, increasing to 52% B in the next 4 min. Then the column was washed for 2.5 min with 90% B and re-equilibrated with 80% A and 20% B for 2 min. Total run time was 9 min.

The mass spectrometer was operated in the positive ion ESI-MS/MS mode. Ion transitions m/z 883.1 > 774.1, m/z 883.1 > 1,047.6, and m/z 883.1 > 960.6 were measured to detect the bST- and Ala-rbST–specific N-terminal peptide with amino acid sequence AFPAMSLSGLFANAVLR, and ion transitions m/z 913.1 > 774.1, m/z 913.1 > 1,047.6, and m/z 913.1 > 960.6 were measured to detect the Met-rbST specific N-terminal peptide with amino acid sequence MFPAMSLSGLFANAVLR, all formed after tryptic digestion (Le Breton et al., 2008). To check the retention time of the N-terminal rbST peptide of interest, a synthetic analog of the rbST peptide was injected at the beginning and end of each series. For the $^{13}C_6{}^{15}N_4$-bST internal standard, the transition m/z 888.1 > 779.1 was followed.

Pretreatment and Measurement of Serum and Milk Samples for Anti-rbST Analysis

Pretreatment of serum and milk samples to detect anti-rbST–induced antibodies was previously described by Ludwig et al. (2012) and is schematically presented

in Supplemental Figure S1 (https://doi.org/10.3168/jds.2020-19209).

Initial In-House Validation

The decision limit (**CCα**) and detection capability (**CCβ**) were determined according to the calibration procedure conforming to Commission Decision 2002/657/EC (European Council, 2002). Milk samples enriched with rbST at 0, 1, 2.5, 5, 10, and 25 ng/mL were processed using the described method. In all runs ,at least 1 calibration curve was included, leading to a total of 17 calibration curves, which were then used to determine CCα and CCβ. Calculation of the concentration was performed by constructing a linear calibration curve of the response factor (peak area ratio of rbST fragment and internal standard) versus the concentration (expressed as absolute amount of rbST protein). The false negative rate β-error, according to the screening criterion of Commission Decision 2002/657/EC (European Council, 2002), was previously determined and described by Ludwig et al. (2012).

RESULTS AND DISCUSSION

Controlled Animal Treatment Experiment

According to the manufacturer's protocol for Met-rbST, optimum milk yields are obtained when rbST treatment starts 57 to 70 d after calving. Following this protocol, Bauman et al. (1999) showed that milk production in dairy cows increased by approximately 10%. Although the treatment in our animal experiment did not start at the prescribed optimal moment, as the study was not set up to determine the effect on the milk production, the effect of the administered rbST hormone on the milk yield was followed. Figure 2 shows that rbST treatment resulted in an increment of the milk yield of, on average, 11%, similar to the findings of Bauman et al. (1999) and de Morais et al. (2017). Overall, the standard deviations due to intraindividual differences are rather high, similar to others reported in the literature (Thornley, 2001).

Analytical Performance of rbST Detection in Serum and Milk

The developed method for the analysis of bST and rbST in milk was based on the previously published method for analysis of rbST in serum (Smits et al., 2015). Therefore, the serum method was adjusted, as described in the Materials and Methods section, and an initial in-house validation as a quantitative confirmatory method according to Commission Decision 2002/657/EC (European Council, 2002) was executed. The analytical performance of the milk method was compared with the original serum method: the decision limit (CCα) and detection capability (CCβ) of both methods were compared.

Table 1. Validation parameters (decision limit, CCα, and detection capability, CCβ) of recombinant bovine somatotropin (rbST) method performance for serum and milk using monolith microcolumns loaded with protein A, tryptic digestion, and liquid chromatography–tandem mass spectrometry analyses

Performance parameter	Serum Met-rbST, ng/mL	Milk Met-rbST, ng/mL
CCα	0.8	2.3
CCβ	1.6	4.5

As shown in Table 1, the CCα of Met-rbST in serum is 3-fold lower than in milk, which most probably arises from interfering lipids in milk, as milk contains on average 3 to 5% lipids (MacGibbon and Taylor, 2006), whereas serum contains less than 0.5% lipids (Raphael et al., 1973). This high fat content of milk is known to block and interfere the antigen-antibody binding during rbST enrichment (Sequeira, 2019). However, because of the straightforward sampling compared with blood, a method to detect rbST use or abuse via milk is preferred.

To enable Ala-rbST detection, sensitivity of the antibody for Ala-rbST was determined and showed to be similar to that of Met-rbST (Heutmekers et al., 2007). Moreover, when a serum sample was spiked with both Met-rbST and Ala-rbST, the binding ratio was, respec-

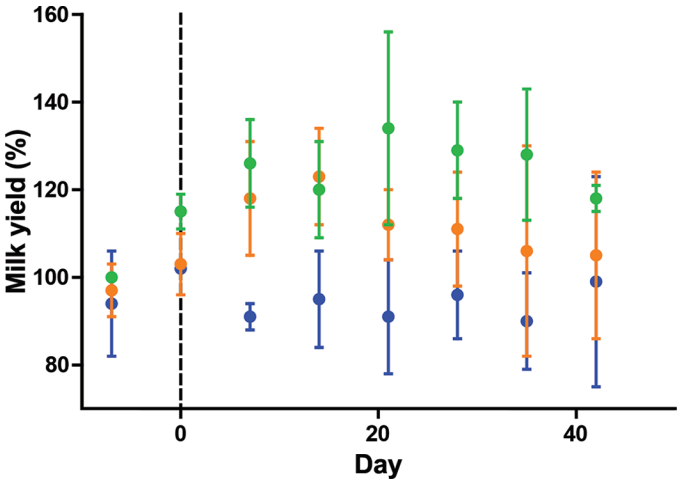

Figure 2. Average milk yield of 10 cows treated with methionine-recombinant bovine somatotropin (Met-rbST, orange dots), 2 cows treated with alanine-rbST (Ala-rbST, green dots), and 2 untreated cows (blue dots). Treatment with Met-rbST and Ala-rbST started at d 0 (dashed vertical line). Milk yield is normalized on the average milk yield before treatment (100%), and error bars represent SD.

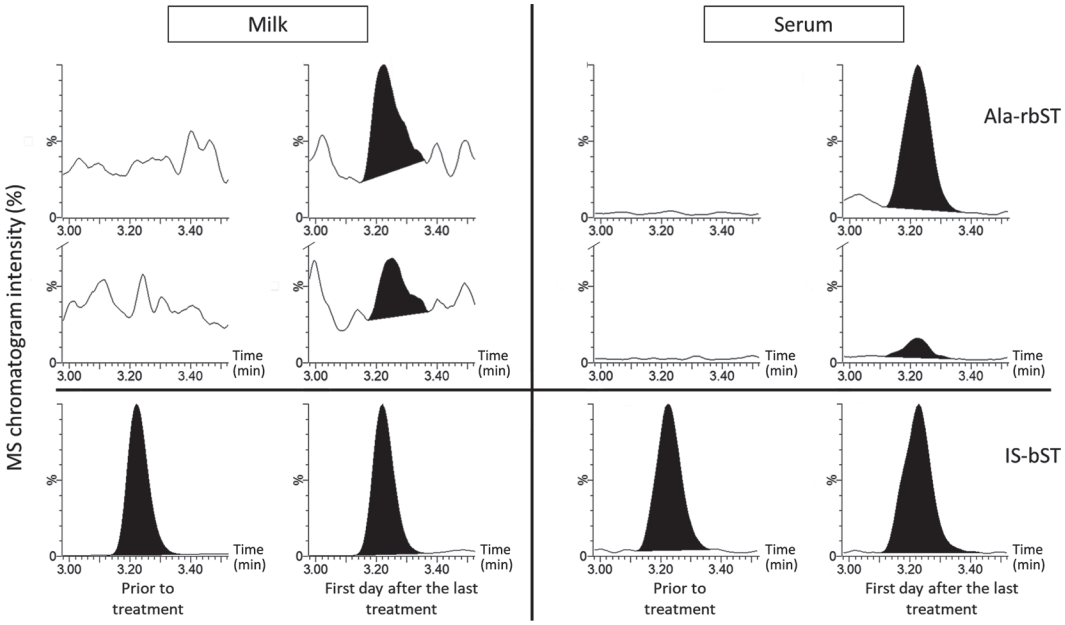

Figure 3. Reconstructed ultra performance liquid chromatography–tandem MS chromatograms [with minor smoothing (2 × 2) using the moving mean (Mn)] of transitions (from top to bottom) m/z 883.1 > m/z 774.1 and m/z 883.1 > m/z 960.6 for the alanine-recombinant bovine somatotropin (Ala-rbST) marker peptide and m/z 888.1 > m/z 779.1 for the methionine-bovine somatotropin (bST) internal standard (IS-bST) of, on the left side, a milk sample taken before treatment and a milk sample taken 1 d after the last Ala-rbST treatment and, on the right side, the corresponding serum samples. All samples were obtained from the same cow.

tively, 6:4, showing antibody affinity for both forms of rbST, even when both forms are present in the sample (data not shown).

Figure 3 shows results obtained from a serum sample and its corresponding milk sample taken 1 d after the first Ala-rbST treatment and a milk sample before treatment. One day after treatment, for both milk and serum, a clear peak is present at the expected retention time of the Ala-rbST peptide marker, demonstrated by the comparison with the internal standard. The peak is missing in the sample taken before treatment. This demonstrates that the Ala-rbST was transferred from blood to milk and that the developed milk method was applicable to incurred milk samples.

Elimination of rbST into Milk

The goal of the present study is to elucidate the elimination of both rbST and anti-rbST from blood to milk. For this, rbST and anti-rbST need to pass the blood-milk barrier. Studying the elimination by measuring both compounds in both serum and milk will yield the required data, enabling development of a control strategy to detect use or abuse of rbST.

Both Met- and Ala-rbST were measured in serum and milk samples, starting just before the first administration. It should be noted that serum and milk samples taken before the rbST treatments did not contain detectable levels of endogenous bST and, obviously, no rbST either. Thus, all detected bST and rbST after the treatments is likely to originate from the corresponding Ala-rbST or Met-rbST treatment.

Figure 4 shows that for both types of treatment (i.e., with Ala-rbST or Met-rbST), a clear increment in serum rbST was observed already the first day after treatment. This increment was seen after each treatment, from the first treatment onward (Supplemental Figure S2, https://doi.org/10.3168/jds.2020-19209). After the last treatment, samples were taken daily for 14 d to study elimination of rbST via milk. Regarding the Ala-rbST treatment, the highest rbST serum concentrations were measured in the first 2 d after treatment, reaching concentrations of about 70 ng/mL (n = 2). Two days after the last treatment, the rbST serum concentrations dropped rapidly. But although concentrations became low, approximately 1 ng/mL of Ala-rbST was still detectable in serum 2 wk after the last treatment. For cows treated with Met-rbST, serum rbST concentrations increased, reaching a maximum 2 d after the last treatment, although this the level was lower compared with Ala-rbST, approximately 30 ng/mL (n = 10). Although lower than the maximum reached with Ala-rbST, Met-rbST serum concentrations stayed elevated for a longer period of time, with

levels above 10 ng/mL (n = 10) 10 d after the last treatment and an average concentration of 3 ng/mL (n = 10) found 2 wk after treatment.

The graph on the right-hand side of Figure 4 shows the measured concentrations of rbST in the corresponding milk samples. Ala-rbST was detected in milk, but in contrast and despite the elevated Met-rbST concentrations present in serum during the 2 wk after administration, Met-rbST concentrations in milk stayed below CCα. The time-dependent Ala-rbST concentration curve in milk is similar to that of Ala-rbST in serum, with the distinction that the detected concentrations in milk are lower. The correlation between milk and serum rbST concentration is dependent on the mechanism that enables rbST to pass the blood-milk barrier. It has been described that endogenous bST (i.e., Ala-bST) is present in the blood and milk, as bST in milk is a growth factor for the calf (Grosvenor et al., 1993). Two routes are considered by which bST that is endogenously present in the mother ends up in her milk. First, bST is transported from the blood circulation via the mammary gland to the milk by transcytosis, similar to the well-studied mechanism for prolactin (Shennan and Peaker, 2000), which belongs to the same hormone family and shows homology with bST (Kelly et al., 1991). Second, it has also been recognized that the mammary gland itself is able to produce bST. However, the exact role and excretion route of mammary bST is not known yet (Neville et al., 2002; Akers, 2006). Because endogenous bST is transported from blood to milk, transport of administered Ala-rbST from blood to milk is also expected. Although this has not yet been demonstrated, this theory of rbST passing the blood-milk barrier is supported by our current observations of Ala-rbST in milk samples after Ala-rbST treatment, showing concentration profiles identical to those in serum. However, despite Met-rbST detected in serum, concentrations of Met-rbST in milk after Met-rbST treatment stayed below CCα. The blood-milk barrier transport mechanism was expected to be similar for both forms of rbST, as they differ by only 1 amino acid. It can be speculated how this difference of 1 amino acid can be responsible for the observed effect that Met-rbST is barely found in milk. Methionine cannot be synthesized by the mammary gland, but methionine is an important part of casein, the major protein component in milk (Bequette et al., 1999; Mabjeesh et al., 2002). It is also known that the mammary gland can use N-terminal-bound methionine of peptides and proteins as a source for its endogenous production of milk proteins, including casein (Wang et al., 1996). Although the proteolytic activity within the mammary gland of cows is not yet fully understood, in humans, breast milk has revealed the presence of a set of peptides with sequential removal of amino acids at the N-terminus, indicating that exopeptidase activity in the mammary gland can be responsible for N-terminal methionine cleavage (Nielsen et al., 2017). If, in the cow's mammary gland, N-terminal methionine is cleaved from the 191-AA-long Met-rbST, then future research should also focus on a 190-AA-long rbST protein that has an N-terminal phenylalanine instead of methionine. The targeted LC-MS/MS measurements in milk should then focus on the N-terminal rbST peptide of the Met-rbST without the methionine (i.e., FPAMSLSGLFANAVLR). In addition, assuming that the injection preparations of Ala-rbST and Met-rbST both contain 500 mg of the recombinant peptide, as declared on the injection preparations, which are legally bound to a strict acceptable deviation of <10% (Fahmy

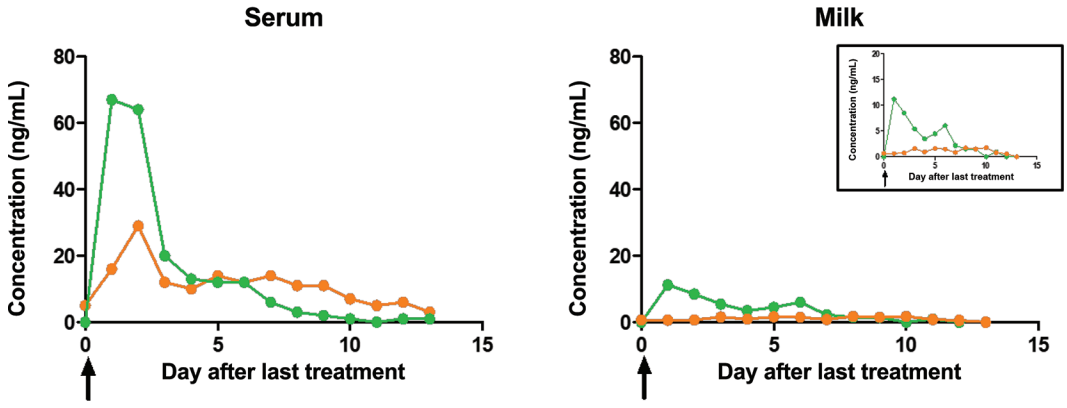

Figure 4. Serum and milk methionine-recombinant bovine somatotropin (Met-rbST, orange dots) and alanine-rbST (Ala-rbST, green dots) concentrations, in ng/mL, as determined by ultra performance liquid chromatography–tandem MS analysis. Arrows mark the moment of the last injection with rbST. The left-hand graph shows average rbST serum concentrations for Met-rbST (n = 10) and Ala-rbST (n = 2); the right-hand graph shows the concentrations in the corresponding milk samples.

Table 2. Validation parameter β-error of anti-recombinant bovine somatotropin antibody-type biomarker for serum, milk, and bulk tank milk samples using flow cytometric immunoassay (Ludwig et al., 2012)

Performance parameter	Serum	Milk	Tank milk
β-error (%)	6	6	≤5

et al., 2013), and the analytical performance was similar for both rbST forms, it was expected that we would find similar amounts of rbST in the serum of treated cows. However, more Ala-rbST was found in serum after treatment than Met-rbST. Because exopeptidases are broadly distributed in the living organism, exopeptidases can cleave the N-terminal methionine peptide when present in the blood (Bradshaw et al., 1998); therefore, also, presence of FPAMSLSGLFANAVLR in serum should be tested in future research.

Elimination of rbST-Induced Antibodies to Milk

Antibodies induced by the injection of rbST were detected in serum samples and their corresponding milk samples using a FCIA (Smits et al., 2012) irrespective of the type of rbST administered. The validation parameter β-error of the FCIA method was described before by Ludwig et al. (2012) and is summarized in Table 2.

Three samples per cow were tested (sample set), one sample before the rbST treatment, one sample 1 wk after the second rbST administration, and one sample 2 wk after the third rbST administration. In total, sample sets of 14 individual cows were tested. Ten cows were treated with the Met-rbST formula, two cows were treated with the Ala-rbST formula, and two cows did not receive treatment with rbST. Figure 5 clearly shows that both rbST treatments result in increased anti-rbST concentrations in both serum and milk (raw data in Supplemental Table S2, https://doi.org/10.3168/jds.2020-19209). Moreover, comparison of the measured sample responses to the decision level enabled us to discriminate between treated and untreated cows in both serum and milk. The presence of antibodies in milk upon both treatments was expected, as both Ala-rbST and Met-rbST will result in an immune response, and a generic Fc-specific transport mechanism from blood toward milk for immunological protection of newborn calves has been reported (Hurley and Theil, 2011).

Although Ala-rbST is fully identical to endogenous bST, cows treated with the Ala-rbST formula showed an immune response by anti-rbST production. In humans, such a phenomenon is also observed when subjects are treated with human growth hormone. Recombinant human growth hormone is also identical to the endogenous form, and antibody formation occurred in 50% of children treated with human growth hormone (Moore and Leppert, 1980). In both cows treated with Ala-rbST, we found antibody formation (Supplemental Table S2, https://doi.org/10.3168/jds.2020-19209). For treatment with Met-rbST an antibody response in 67% of the cows was found, as previously described (Ludwig et al., 2012). The immune responses in these cows are likely due to formation of rbST aggregates in the formulas (Rosenberg, 2006; Moussa et al., 2016).

To pinpoint rbST use or abuse for control purposes, rbST-induced antibodies can be detected in both serum and milk from treated cows. Milk samples are preferred, as milk sampling is noninvasive. However, because not all cows will show an immune response upon treatment, for control purposes multiple cows should be sampled, or, even better, samples should be taken from the farmer's milk storage tank, as it has been shown that taking samples from tanks for detection of rbST-induced antibodies is more adequate than sampling from individual cows, resulting in a true positive rate of over 95% for tank milk (Ludwig et al., 2012).

Strategy for rbST Enforcement Purposes

The elimination patterns for rbST and the rbST-induced antibodies, as presented in this study, show that, for enforcement purposes, the detection of rbST-induced antibodies in tank milk can serve as an antibody-type

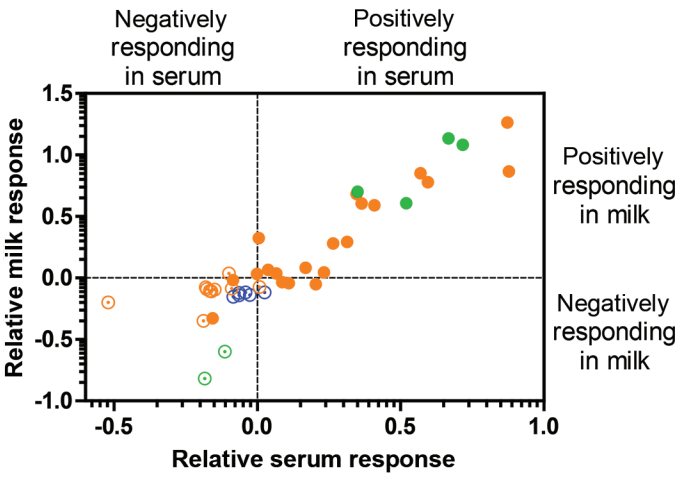

Figure 5. Recombinant bovine somatotropin (rbST) antibody relative responses in corresponding serum and milk samples. Measured flow cytometric immunoassay fluorescence signals are normalized according to Smits et al. (2012). For analysis, samples from each cow at the following time points were used: 1 wk before treatment (⊙) and 3 and 6 wk after start of treatment (●). Samples are taken from 2 untreated (blue ⊙) cows, 10 cows treated with the methionine-rbST formula (orange ⊙, ●), and 2 cows treated with the alanine-rbST formula (green ⊙, ●). Dashed lines represent the decision level to discriminate between treated and untreated cows for, respectively, serum and milk.

biomarker to screen for rbST administration. Ideally, results from tank milk analysis need to be available within 1 d. When screening results suggest rbST use or abuse, blood samples need to be drawn immediately for subsequent confirmatory serum analysis by LC-MS/MS. These analyses are needed to confirm presence of rbST in the cow and to identify whether Ala-rbST or Met-rbST was used.

CONCLUSIONS

Investigating the elimination of rbST, the transfer from blood to milk, demonstrated that the blood-milk barrier for rbST is very specific. Both rbST formulations are detectable in serum, but Met-rbST was barely transferred to milk. As expected, Ala-rbST, which is similar to endogenous bST, was transferred to the milk, and the levels were about 5 times lower than those in the serum samples. Thus, a difference of 1 amino acid (methionine) makes an important difference for elimination into milk. Regarding the immune response, both formulations resulted in a clear response and similar levels of anti-rbST in serum. And as expected, these anti-rbST antibodies were transferred to milk. The present study thus demonstrates that, for control purposes, screening for rbST use and abuse can easily be performed by measuring anti-rbST antibodies as an antibody-type biomarker in tank milk. However, subsequent LC-MS/MS analyses of serum samples are needed to confirm the type of rbST used, as Met-rbST is not able to cross the blood-milk barrier.

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