

A LC-MS-MS method to detect recombinant bovine somatotropin misuse in buffalos

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Abstract Recombinant bovine somatotropin (rbST) is a peptide hormone used to increase milk yield in cows and buffalos. In Europe, its use has been banned. However, rbST is sometimes illegally included in zootechnical practices for profit purposes, undermining the fair trade and the law prescriptions. For this reason, efficient and reliable analytical techniques are required to contrast rbST misuse. A few LC-MS-MS methods have been developed to detect, in cow serum, methyonil-rbST, one of the two main rbST forms available on the market. The other form, which is widespread, is identical to the most abundant variant of bovine somatotropin (bST) and differs from the buffalo somatotropin for one amino acid in the N-terminus. For this reason, it is technically possible to distinguish both rbST forms in serum of buffalos. In this work, we describe a novel LC-MS-MS-based method, capable to quantify, with a high sensitivity and selectivity, the methyonil-rbST and the other bST-identical recombinant form in buffalo serum, previously purified using a solid-phase extraction procedure. The method was internally validated and used to analyse 152

serum samples, collected from eight buffalos administered with rbST for a period of 3 months, according to conventional protocols. The obtained results confirmed the suitability of the method in the detection of illegal hormonal treatments.

Keywords Recombinant bovine somatotropin (rbST) · High performance liquid chromatography · Mass spectrometry · Buffalo

Introduction

The problem of illegal or undeclared use of recombinant bovine somatotropin (rbST) in animal productions is an issue involving not only cow breeding, but also buffalos. In fact, rbST is a peptide hormone with a high identity with the pituitary somatotropin (≈ 99 and 100 %), whose function consists in the stimulation of galactopoiesis [1], increasing the milk yield in both cows [2–4] and buffalos [5–7].

The buffalo breeding is a well-established activity in some area of the world, where climate conditions are less suitable for bovines, like in South and Southeast regions of Asia, or where the dairy products obtained from milk of this species are traditionally much appreciated, like in some Mediterranean Countries [8]. Furthermore, a growth of business related to buffalo breeding is a reality also involving South America [9].

The use of somatotropin for zootechnical purposes is allowed in some countries of the world, such as the USA, but forbidden in other, such as the member states of the European Union, which banned treatments with this hormone in livestock with the Decision 1999/879/CE [10]. However, although the Joint FAO/WHO Expert Committee on Food Additives (JECFA) concluded that the use of these drugs

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according to good practice in veterinary medicine did not represent a dietary hazard to human health, reaffirming its previous decision on acceptable daily intake (ADI) and maximum residue level (MRL) values as ‘not specified’ for rbSTs [11], issues regarding the obligation to declare rbST employment, where allowed, and the illegal use of the hormone, where forbidden, are still unsolved concerns.

In this frame, scientists are carrying out studies to develop and validate analytical tools to be used in official controls, in order to ensure the compliance with applicable laws and safeguard the consumers’ rights. Other than screening methods, which may involve investigation on possible suitable biomarkers [12, 13], direct detection of the recombinant hormone may involve rapid and inexpensive immunochemical methods [14–16] or more performant techniques, such as liquid chromatography coupled to mass-spectrometry (LC-MS-MS), as recently reviewed by Dervilly-Pinel et al. [17]. The latter is the basis of some confirmatory methods developed to identify methionyl-rbST, one of the possible recombinant forms existing on the market, and successfully applied to serum samples from rbST-treated bovines [18–20]. Unfortunately, one other recombinant form of somatotropin exists and it is widely spread both in the legal and in the illegal market. Such molecule is identical to the most abundant bovine variant produced by the pituitary gland, and as a consequence, it cannot be detected by using conventional analytical techniques. However, the major pituitary form of the buffalo somatotropin and the two previously described rbSTs differ respectively for two or one amino acids at the level of the N-terminus of the protein (Fig. 1). This small difference makes both the recombinant peptides eventually distinguishable from the naturally produced ST variants.

In this study, we describe a novel analytical method based on LC-MS-MS, able to identify both the recombinant forms of somatotropin, which was used for the analysis of samples from buffalos treated with rbST, for a period of 3 months.

Materials and methods

Reagents and materials

Lyophilised highly purified recombinant methionyl bovine ST (mrBST) was purchased from the National Hormone & Peptide Program (NHPP), Harbor-UCLA Medical Center (Torrance, CA, USA); recombinant bST was extracted from the slow release formulation (Boostin[®], 500 mg/dose from LG Life Science, Seoul, Korea); lyophilised recombinant porcine ST (rpST), human serum albumin (HAS) and reagents for buffers were purchased from Sigma-Aldrich (St. Louis, MO, US). Sequence grade modified trypsin (EC 3.4.21.4) was purchased from Promega (Promega, Madison, WI, USA). All solvents and additives for HPLC-MS-MS runs (acetonitrile,

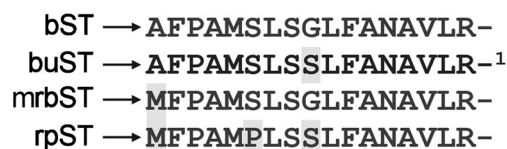


Fig. 1 Primary sequence of the N-terminus tryptic fragments of the most abundant variants of pituitary bovine ST (bST), which is identical to the recombinant form produced by LG-Lifesciences (subsequently called rbST) (MW 1765.10). Buffalo ST (buST) differs from bST for Ser in position 9 (MW 1795.13); methionyl-bST (mrBST) for Met in position 1 (MW 1825.22) and recombinant porcine ST (rpST) for Met in position 1, Pro in position 6 and Ser in position 1 (MW 1865.28). Differences with respect to bST are highlighted in *grey*. ¹buST sequence may present other variants. This is the sequence of Mediterranean buffalo, as verified by a previously performed DNA analysis carried out in our laboratory (data not shown)

methanol, water and formic acid) were HPLC-MS grade from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of standards

Stock solutions of rbST, mrBST and rpST for preparation of calibration curves standards (CC) and quality control (QC) samples were prepared separately. CC standards were prepared by diluting the rbST-extract primary solutions of (see ‘Instrumentation’) in a solution of 0.1 mg/ml human serum albumin (HSA), 0.05 M phosphate buffer, pH 7.3, until a final volume of 20 µl each standards. HSA was run in HPLC-MS/MS to verify the absence of any interference.

Methionyl rbST was weighted, and primary stock solution of 1 mg/ml was prepared. Concentration was confirmed by spectrophotometric analysis, and CC standards were made as described for rbST.

Porcine rbST primary solution for internal standards (IS) was made as described by the manufacturer, and single working stocks of 50 ng/ml were prepared in a final volume of 20 µl by proper dilution in 0.1 mg/ml human serum albumin (HSA), 0.05 M phosphate buffer, pH 7.3. After preparation, all the standards were frozen at –80 °C.

For analysis, all frozen samples collected from animals, CC, QC samples and IS were thawed at room temperature, added to serum samples and thoroughly mixed before processing.

Instrumentation

Instrument layout included an UHPLC system, constituted by a binary pump, a column oven, an autosampler outfitted with Peltier tray (all of them 1290 Series, Agilent Technologies, Santa Clara, CA, USA) and a quaternary HPLC pump (Series 200, Perkin Elmer, Boston, MA, USA) coupled to an AB-Sciex API 4000 triple quadrupole mass spectrometer (Concord, Ontario, Canada) and equipped with a Turbo-V IonSpray source. A ten port switching valve (Valco

Instruments Co. Inc., Huston, TX, USA) was used as a divert valve. HPLC separations were performed by a 2.1×150 mm, 3 μ m particle size ACE Excel 3 C8 column (Advanced Chromatography Technologies, Aberdeen, UK).

Recombinant bST extraction

Recombinant bST was extracted from syringes containing a slow release formulation of the hormone (Boostin[®], LG-Lifescience, Seoul, South Korea). In order to separate the grease layer (upper layer) from the protein solution, 500 μ l of the preparation was mixed with 9.5 ml of carbonate buffer (40 mM NaHCO₃, pH 9.6), vortexed until complete mixing with buffer and then centrifuged at $10,000 \times g$, 15 min, 4 °C. After centrifugation, 1 ml of the protein solution was collected and mixed again with 9 ml of carbonate buffer, gently vortexed and centrifuged as previously described. Two millilitres of the solution was then collected, and the protein concentration was measured on the basis of 280 nm absorbance (extinction coefficient: $13,610 \text{ M}^{-1} \text{ cm}^{-1}$) using a Nanodrop[®] ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA).

The eventual presence of a detectable amount of other contaminant proteins in rbST final solution was evaluated by SDS-PAGE. Gels were stained with Coomassie blue according to standard protocols.

Animal treatment with rbST and sample collection

Sixteen Mediterranean Italian buffalos were selected from the Animal Production Research Centre of Tormancina (Italy) and were managed in standard conditions with ad libitum feeding and fresh water always available. The animals were randomly allotted to two groups. One group was assigned to the rbST treatment ($n=8$) and the other served as the controls ($n=8$).

Treatment was performed by administering a sustained release formulation of rbST (Boostin[®], LG-Lifescience, Seoul, Korea) (500 mg in 2 ml of vehicle) by subcutaneous injections in the tailhead area. Controls were injected with a saline physiological solution. The treatment was performed over a period of 12 weeks, starting from the 10th week postpartum, for a total of six (named A, B, C, D, E and F) biweekly cycles of administration (one rbST injection every 14 days, as required by the standard zootechnical protocol).

Blood was collected via venipuncture of the jugular vein before the first meal of the day, the day before the onset of treatment (day -1) and then twice a week on days 2, 5, 9 and 14 after each rbST administration in cycles A and D. Blood was also collected in cycle F (last rbST injection cycle) on days 1, 2, 3, 5, 7, 9, 11, 14, 16 and 28 in order to obtain more precise indication on rbST kinetic.

A total number of 152 samples were analysed. After withdrawing, blood was allowed to clot at room temperature and

then centrifuged at $2200 \times g$ for 20 min at 4 °C. Sera were divided into aliquots and stored at -80 °C until analysis.

Recombinant bST purification from serum for MS analysis

For rbST purification from serum, the method proposed by Le Breton et al. [18] was modified.

In order to understand if SPE columns with different characteristics determined an improvement of rbST recovery, extractions were carried out on cartridges C4, 20 μ m particles and 300 Å pore (Upti-Clean WC4-500MG/6ML, Interchim, Montlucon, France) ($n=8$); C4, 45 μ m particles and 500 Å pore (Supelclean[™] LC-4 500 mg/3 ml, Supelco, Bellefonte, PA, USA) ($n=8$); and Phenyl, 40–60 μ m particles and 60 Å pore (HyperSep ph 500 mg/6 ml, Thermo Scientific, Waltham, MA, USA) ($n=6$). In particular, since selection of the type of column preceded the selection of the optimal bed volume, trials were carried using a bed of 500 mg, instead of 200 mg, and 4 ml serum, as suggested in the original protocol. Serum of non-treated buffalos in late lactation was fortified with 500 ng/ml rbST before extraction. The residual rbST concentration was measured by ELISA, according to Castigliego et al. [14], after pellet resuspension in 200 μ l in phosphate buffered saline (15 mM Na₂HPO₄, 5 mM NaH₂PO₄, 116 mM NaCl, pH 7.3), with 0.05 % tween 20 (PBS-T), and further dilution 1/10 in the same buffer. In order to verify the efficiency of different extraction protocols, the total protein concentration after extraction was measured on the basis of 280 nm absorbance (extinction coefficient $13,610 \text{ M}^{-1} \text{ cm}^{-1}$) using a Nanodrop[®] ND-1000 spectrophotometer and rbST concentration was measured as already described. Eight measurements were carried out for the protocol with and eight for the protocol without the addition of ammonium acetate after elution of the proteins.

The final protocol is reported below: After thawing at room temperature, serum samples were mixed, centrifuged 5 min at $13,000 \times g$, in order to pellet every possible clot leftover. Serum (1.2 ml) was then transferred in beakers and spiked with 20 μ l of IS solution. Phosphate buffer 0.1 M (5.4 ml), pH 6.9 was then added to sera. In conditions of continuous stirring, 5.4 ml of a saturated solution of ammonium sulphate was added drop by drop, in order to reach a final ammonium sulphate concentration of 45 %. The solution with the protein precipitate was then left some minutes to stir, then transferred to 15-ml polypropylene tubes and left overnight at 4 °C. After a centrifugation at $2700 \times g$, 4 °C for 30 min, the supernatant was discarded and the pellet was resuspended in 1.8 ml of 0.1 M phosphate buffer, pH 6.

Each sample solution was then let to flow through a 200-mg C4 SPE cartridge (Upti-Clean WC4-200MG/3ML, Interchim, Montlucon, France) previously activated with 4 ml of methanol and subsequently with 4 ml of 0.1 M

phosphate buffer, pH 6. The columns were then washed in sequence with 2 ml of water, 0.1 % TFA and 2 ml of a solution 30:70 (v/v) acetonitrile/H₂O, 0.1 % TFA. Recombinant bST was then eluted with 3 ml of a solution 80:20 (v/v) acetonitrile/H₂O, 0.1 % TFA and collected in 15 ml polypropylene tubes. To each tube, 3 ml of ice-cold methanol was added and left at -20 °C for 30 min. One hundred twenty microlitres of 1 M ammonium acetate was then added. After further 90 min at -20 °C, the tubes were centrifuged at 2700×g, at -10 °C for 30 min. The supernatant was then carefully discarded. Tubes were left capsized on a sheet of absorbent paper for 15 min in a stove at 37 °C. The pellet was then thoroughly resuspended in 150 µl of 50 mM ammonium carbonate, 5 mM EDTA, pH 8 and subsequently transferred into a 1.5-ml polypropylene microcentrifuge tube. Three micrograms of trypsin was then added and left overnight at 37 °C for digestion in a water bath. The tube content was then dried at 40 °C under a gentle stream of nitrogen, reconstituted by 50 µl of a solution 20:80 (v/v) acetonitrile/H₂O, 1 % formic acid and vortexed for 10 min. The suspension was then centrifuged 10 min at 13,000×g before transferring 35 µl of the supernatant to HPLC vials for the injection.

HPLC conditions

Binary pump system, quaternary pump and divert valve were properly coupled in order to discard both head and tail of the HPLC runs. This granted an adequate robustness to the analytical method. Of each sample, 20 µl was injected in the HPLC-MS-MS apparatus, and HPLC separation was carried out by the settings listed in Table 1, while divert valve worked as reported in Table 2.

Mass spectrometry conditions

A positive ion mode selected reaction monitoring (SRM) method was set up, in order to quantify the compounds of

interest. Two transitions were monitored for each compound, making use of optimized declustering potentials (DPs), collision energies (CEs) and collision exit potentials (CXPs), as listed in Table 3. Further operative parameters were set as follows: IonSpray Voltage (ISV), 5.5 kV; Gas Source 1 (GS1), 50; Gas Source 2 (GS2), 60; turbo temperature (TEM), 650 °C; entrance potential (EP), 10 V; IQ1 lens potentials (IQ1), -10.4 V; collision (CAD) gas, nitrogen; operative pressure with CAD gas on, 5.5 mPa.

Within-lab validation

The method was internally validated taking into consideration the dispositions of Decision 2002/657/EC [21] concerning the performance of analytical methods and the guidelines on bioanalytical method validation of the European Medicine Agency [22].

The following parameters were assessed: recovery, elution time variability, lower limit of detection (LLOD), lower limit of quantification (LLOQ), within-run and between-run coefficient of variation, specificity, stability of the analyte in the sample and carryover.

Recovery was determined by comparing spiked serum extracts with unextracted standards. Six replicates of spiked serum samples were used at the concentrations of 1, 10 and 50 ng/ml. The percentage of recovery was calculated on the basis of the ratio between the peak area of the spiked samples and those of pure analyte solutions. The recovery of the internal standard was calculated at the single concentration of 50 ng/ml.

To test performance of HPLC separation, the relative retention time of the analytes, namely the ratio of the chromatographic retention time of rbST or mrbST to that of the internal standard (rpST), was compared to those of the calibration solutions. Such a ratio should be less than 2.5 %, according to the Commission Decision 2002/657/EC [21].

Table 1 HPLC pumps program. HPLC quaternary pump was used to supply mass spectrometer with flow when the eluent from HPLC binary pump, used for the analytical separation, went to the waste

Step	HPLC quaternary pump				HPLC binary pump			
	Total time (min)	Flow rate (µl/min)	Methanol (%)	Water (%)	Total time (min)	Flow rate (µl/min)	Solvent A ^a (%)	Solvent B ^b (%)
0	0.0	100	50	50	0.0	500	0	100
1	17.3	100	50	50	2.0	500	0	100
2					12.0	500	90	10
3					14.3	500	90	10
4					14.4	500	0	100
5					17.3	500	0	100

^a Solvent A: acetonitrile (80 %) and methanol (20 %) + formic acid (0.1 %)

^b Solvent B is water + formic acid (0.1 %)

Table 2 Divert valve program

Switching valve	
Total time (min)	Position
0.0	A
4.5	B
9.5	A

A three time signal-to-noise ratio at the time window in which the analyte was expected was used as the lowest level of fortification to calculate LLOD and LLOQ. Ten blank sera were fortified with 0.25, 0.5 and 1 ng/ml of analytes. LLOQ was then calculated as the smallest amount of analytes detectable with a SD (standard deviation) <20 %. LLOD was calculated as the smallest concentration to which at least 50 % of the fortified samples where detectable.

The within-run coefficient of variation was calculated by analysing six replicates at three different levels: 1 (LLOQ), 10 and 50 ng/ml and expressed as ratio between SD of the measurements and means. The between-run coefficient of variation was determined by repeating the measurements in three different runs and expressed as the SD of the means on the three repeated runs and their grandmean.

Specificity was tested by analysing 20 blank samples. Possible interferences in the region corresponding to the retention time of the analytes were checked. Furthermore, blank samples were fortified with a total amount of 1000 ng of buST extracted from the pituitary glands according to the protocol proposed by Wood et al. [23]. The concentration of buST in the final extract solution was measured by ELISA, as described by Castigliero et al. [24].

The presence of peaks or unusual signals in the region of interest was evaluated.

Due to the fact that the amount of single sera was limited to cover all the three storing conditions and that previous experiences with pooled sera resulted in altered results, even for freshly added standards, the stability of the analytes in serum samples was tested by fortifying three aliquots of three different serum samples with 10 ng/ml of rbST or mrbST.

Table 3 Mass spectrometry operative parameters

Analyte	SRM transition	Operative parameters		
		DP	CE	CXP
rpST	934.8 → 169.2	103	62	3.6
	934.8 → 794.6		38	5.8
rbST	883.9 → 120.2	99	103	2.8
	883.9 → 169.2		62	3.9
mrbST	913.4 → 70.0	87	100	4.9
	913.4 → 774.4		37	21.5

The three groups were stored at different temperature conditions for 1 week after fortification: the first at RT, the second at 4 °C and the third was frozen and thawed for three times. After the period of storing, the samples were processed and compared with samples spiked soon before processing. The IS was freshly added to all the samples before analysis.

To assess the stability of the IS, the same procedure was adopted, but rpST was added before storing and rbST was added fresh soon before processing (50 ng/ml). In this case, rbST was used as internal standard. The concentration of the analytes was expressed as percentage of the ratio measured analyte/fresh added analyte.

Carryover was assessed by injecting blank samples after fortification with 250 ng/ml (n=3) and 1000 ng/ml (n=3), corresponding to one and four times the highest concentration of calibration standards, respectively.

Calibration curves were made using five standard solutions of analytes plus blank: 1 (LLOQ), 2, 10, 50 and 250 ng/ml. The analytes were added to blank sera and then processed together with true samples. Each point was run in duplicate. Curves were built by plotting the analyte/IS peak area ratio against the nominal analyte concentrations and fit by least square linear regression using a weighting factor of 1/X.

Results and discussion

Optimization of rbST extraction from serum

The SPE method reported by Le Breton et al. [18], the first procedure proposed for rbST purification from serum, was used as a basis and modified to obtain the highest rbST yield possible. Columns with different characteristics were tried, but the more performant were those with a C4 matrix active group, with particle size of 20 µm and 300 Å pore. Even similar products, with particle size of 45 µm and 500 Å pore size, performed noticeably worse, with a final rbST yield of about one half. Smallest pore size (60 Å) and a slightly different chemistry of bonding (phenyl groups) resulted in a poor recovery (Table 4).

One important variation to the original method was the removal of a long step related to nitrogen drying after elution. In fact, the direct addition of cold methanol to the eluate, reduced in volume, was sufficient to obtain a good level of sample cleanness. In order to improve the yield of precipitated proteins during the incubation at -20 °C, the pH of the solution was increased by adding ammonium acetate. This allowed a more efficient protein precipitation, likely due to increased capability of protein-protein interaction. In fact, in strongly acidic conditions, proteins are sharply positively charged and then more subjected to reciprocal repulsion, while, by raising pH, the net charge of the proteins decreases and repulsive forces are less influential. A better recovery of

Table 4 Residual rbST amount (measured by ELISA) after extraction using different SPE columns. A. C4, 20 μm particles and 300 \AA pore ($n=8$); B. C4, 45 μm particles and 500 \AA pore ($n=8$); C. Phenyl, 40–60 μm particles and 60 \AA pore ($n=6$)

	A	B	C
rbST residual amount (%)	35.1	26.5	nm
of pre-extraction amount)	28.2	16.4	6.2
	37.6	15.9	4.2
	42.3	18.6	nm
	38.8	25	nm
	41	14.2	2.1
	45.4	13.5	
	37.8	25.1	

nm non-measurable

the proteins eluted from the column was demonstrated by measuring the total proteins concentration after resuspension of the pellet (final step before addition of trypsin), which was about 1.36 ± 0.15 mg/ml, against concentrations, in our hands, under the measurable threshold of the spectrophotometer, when ammonium acetate was not added.

The considerable amount of proteins obtainable with this expedient allowed us to choose SPE column with a lower capacity (200 mg instead of 500 mg) and reduce the needed amount of serum to only 1.2 ml. Reducing agents, such as dithiothreitol, were not included in the digestion buffer, as it did not produce any improvement in the mass spectrometric signal (data not shown). The high concentration of formic acid in the reconstitution buffer was necessary to allow an efficient reconstitution after prior the injection.

Optimization of HPLC-MS-MS

Mass spectrometry method was based on selected reaction monitoring (SRM). The optimization of the instrumental parameters was carried out by setting both compound parameters and source parameters. As far as the choice of the SRM transition is concerned, several transitions of each compound were optimized and monitored: Two of them were selected as a quantifier and as a qualifier transitions based on their stability, intensity and signal-to-noise ratios in real samples. In particular, among the stable transitions, the quantifier had the best signal-to-noise ratio, while the qualifier exhibited the second-best signal-to-noise ratio. For rpST, the transition was $933.8 \rightarrow 794.6$ (quantifier) and $933.8 \rightarrow 169.2$ (qualifier), for rbST was $883.9 \rightarrow 169.2$ (quantifier) and $883.9 \rightarrow 120.2$ (qualifier) and for mrbST was $913.4 \rightarrow 774.4$ (quantifier) and $913.4 \rightarrow 70.0$ (qualifier).

The HPLC method was set up in order to separate the analytes of interest from some interferences, which significantly affected the measurements. The resulting run was robust, reliable and allowed a good accuracy. As an example, some HPLC-MS-MS chromatograms are reported in Fig. 2.

Method performance

All the performance outcomes, used for internal validation, are reported below and summarized in Table 5.

The relative recoveries for rbST, mrbST and rpST were comparable, as expected, due to the fact that the three molecules have a high identity degree. No marked differences were observed for the three different concentrations at which trials were carried out (Table 5). Despite the loss of analytes during the process of extraction was quantitatively considerable, the sensitivity of the method was still good and the method was compliant with EU requirements as for performance, as showed later on.

The retention times (min) measured for rbST, mrbST and rpST were 8.58 ± 0.03 , 8.73 ± 0.03 and 8.84 ± 0.03 (mean \pm SD), respectively.

The ratio of the chromatographic retention time of rbST and mrbST peptides to that of the internal standard (rpST peptide) had a variation lower than 2.5 % with regard to that of the calibration solutions, as prescribed by EU requirements, with highest discrepancy of 0.4 % for rbST and 0.2 % for mrbST.

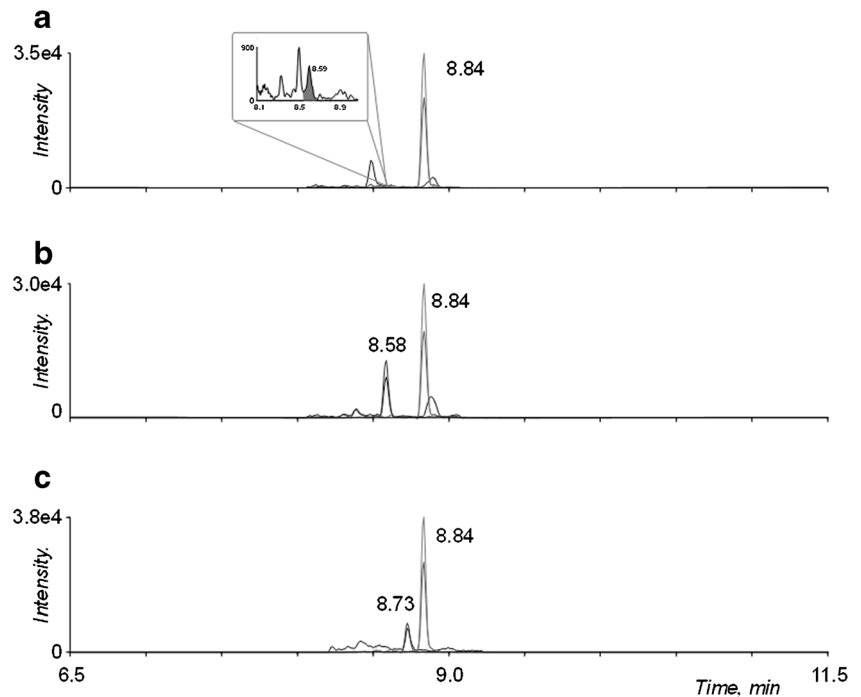
LLOD and LLOQ were determined to be 0.25 and 1 ng/ml, respectively, for both rbST and mrbST. Such a low LLOQ allows measurements of small amounts of residual recombinant hormone in serum, permitting to rely on a more extended window during which the presence of the molecule can be detected, within each biweekly drug administration cycle.

The percentage of within-run variability calculated in three repeated trials for three different analyte concentrations was, as expected, higher for the lowest concentrations (LLOQ). However, it was never found to be above 20 % in both rbST and mrbST. At the same time, between-assay CV was always lower than 15 % for the three concentration, as suggested by the Guideline on bioanalytical method validation of EMEA [22] (Table 5).

Specificity was tested by analysis of blank samples ($n=20$), for which no interference was detected at the time the target analytes were expected to elute (from about 8.45 to 9 min). Due to the fact that the tryptic N-terminus peptide from buST has a high identity with to N-terminus peptides from rpST (Fig. 1) used as internal standard, rbST and mrbST, which are the analytical targets, samples fortified with a high amount of buST were also tested. In the region corresponding to the retention time, no detectable differences with blank samples were observed. This demonstrates that the MS/MS method is not sensitive toward buST.

Stability of the analytes in the samples was determined after measurement of rbST, mrbST and rpST in fortified blank sera, left in different temperature conditions. Only the samples left at RT for 1 week showed a marked decrease of detectability for all the three analytes, while a difference lower than the between-run CV was observed for samples left 1 week at 4 $^{\circ}$.

Fig. 2 Mass Spectrometry chromatograms from (A) a real sample containing rbST (8.58 min) at a concentration level close to LLOQ, (B) a real sample containing rbST at a medium concentration level (22 ng/ml) and (C) a real serum spiked with 10 ng/ml of mrbST (8.73 min). All the chromatograms show the peak of rpST at 8.84 min



Samples spiked and refrozen showed a comparable recovery with the fresh prepared samples since the difference cannot be considered as significant due to the fact that it was lower than the percentage of within-assay variability (Table 5). The relative stability at 4 °C and after cycles of thawing and refreezing may be important in case shipment conditions undergo some interruption in the freeze chain.

The rbST and mrbST concentration used to measure possible carryover corresponded to about one and four

times the highest concentration of the calibration curve. For concentration of 1000 ng/ml, far from the possible expected concentration of recombinant somatotropin in serum, values of carryover were greater than LLOQ. For concentration corresponding to the upper point of the calibration curve, the measured carryover was less than one half the LLOQ (Table 5).

Standard curves were found to have a R^2 always higher than 0.99, showing a good linearity.

Table 5 Performance parameters for rbST, mrbST and rpST (IS)

		rbST	mrbST	rpST
Recovery (%)	1 ng/ml	27.2	29.6	
	10 ng/ml	33.1	27.8	
	50 ng/ml	29	30.1	
	50 ng/ml			32.7
LLOD (ng/ml)		0.25	0.25	
LLOQ (ng/ml)		1	1	
Within-run CV (%)	1 ng/ml	17.3	18.2	
	10 ng/ml	13.9	10.2	
	50 ng/ml	6.2	11.8	
Between-run CV (%)	1 ng/ml	15.2	17.6	
	10 ng/ml	14.4	14.1	
	50 ng/ml	5.1	13.2	
Stability after 1 week (% of fresh samples)	RT	54.2	48.2	66.2
	4 °C	106.8	93.2	85.4
	Freezing/thawing (3 cycles)	103.1	101	95.1
Carryover (% LLOQ)	1000 ng/ml	195	224	
	250 ng/ml	32.3	38.5	

Analysis of sera from treated animals

The analysis of sera confirmed the suitability of the method for identification of buffalos treated with rbST. In fact, rbST was detected in more than one half of samples, with highest concentrations in the first days after each drug administration and peaks up to about 250 ng/ml (Table 6).

Recombinant bST levels decreased quite quickly after the peak/plateau of the first days, becoming, in a number of cases, already undetectable on day 9. From day 11 on, the rbST concentration was almost always under the minimum detection level. To note, the sporadic and exceptionally low rbST values are detected in some samples collected during the first days after drug injection when the concentration of the hormone is expected to be high. This occurrence was already noticed in a study performed on cows by Castiglieo et al. [24], where rbST serum content was measured by ELISA, and previously reported by Cisse et al. [25]. The reasons, as suggested by these authors, may be a variability of clearance rate during the day or a delay in releasing the active molecule.

This pharmacokinetic pattern is essentially over impossible to those reported by Castiglieo et al. [24] in cows and Castiglieo et al. [5] in buffalos obtained by analysing with ELISA serum collected from animals treated according to a

similar experimental protocol. To be noticed, the presence, on an average, is of very high levels.

Patterns associated to some animals included in this study are not so different from those reported by Smith et al. [20] characterized by a decrease of rbST concentrations along the biweekly cycle of hormone administration, with a maximum concentration of around 20 ng/ml and rbST. Le Breton et al. [18], who performed a pharmacokinetic study in a period of about 4 days after the hormone administration, observed a rapid increase of ST in the first hours and a slight decrease after 20 h, but have not found ST levels above 10 ng/ml.

Le Breton et al. [19], in a different study, performed by analysing by HPLC-MS/MS sera from two cows treated with methionyl rbST, found high concentrations in sera collected after 1 day from the drug administration, overcoming 100 ng/ml in one case and around 40 ng/ml in the other. The authors observed a different behaviour in the drug kinetic of the two animals, with either a marked or a more sustained decrease in ST concentration, with the analyte detectable still after 13 days. Unfortunately, direct comparisons with the present study cannot be carried out, beyond the difference in animal species, due to the fact that, in the study of Le Breton et al. [19], two sequential hormone injections were performed.

However, differences in ST kinetics found in and between the previously mentioned studies, even when compared to the

Table 6 rbST concentration (ng/ml) of sera collected from experimentally treated buffalos (cycles A, D and F) and analysed by the method developed in this study

Cycle	Day	Buffalo							
		a	b	c	d	e	f	g	h
A	-1	<LLOD	<LLOD	<LLOD	<LLOD	<LLOD	<LLOD	<LLOD	<LLOD
	2	248.9	0.8	60.4	143.7	2.1	126.8	41.6	65.8
	5	1.4	95.9	10.9	6	7	nq	12.6	148.2
	9	5.7	13	1.45	<LLOD	<LLOD	<LLOD	<LLOD	2.2
D	14	<LLOD	<LLOD	nq	<LLOD	<LLOD	<LLOD	<LLOD	<LLOD
	2	22.6	12.4	22.2	17.4	28.3	27	22.6	11.9
	5	1.5	1.3	2.2	1.5	1.3	3.4	2.4	11.9
	9	nq	nq	nq	nq	nq	nq	nq	nq
F	14	<LLOD	<LLOD	<LLOD	<LLOD	<LLOD	<LLOD	<LLOD	<LLOD
	1	105.2	46.8	218.5	68.3	100.3	30.5	142.4	1.2
	2	39.9	19	65.3	24.8	24.6	26.9	28.6	24.8
	3	6.2	5.9	11.4	5.5	9.9	6.5	6.9	6
	5	2.4	4.3	nq	2.9	3.8	1.6	2.8	4.2
	7	1.8	2	nq	1.5	2.2	1.4	1.6	nq
	9	1.25	<LLOD	1.2	2	2.2	nq	11.3	nq
	11	<LLOD	<LLOD	<LLOD	nq	nq	<LLOD	<LLOD	<LLOD
	14	<LLOD	<LLOD	<LLOD	<LLOD	<LLOD	<LLOD	<LLOD	<LLOD
	16	<LLOD	<LLOD	<LLOD	<LLOD	<LLOD	<LLOD	<LLOD	<LLOD
28	<LLOD	<LLOD	<LLOD	<LLOD	<LLOD	<LLOD	<LLOD	<LLOD	

nq nonquantifiable (values comprised between LLOD and LLOQ)

present study, cannot be only attributed to the characteristic of the molecule or the composition of the hormone preparations used, which might lead to a different release rate of the hormone, but rather suggest the existence of a great level of variability between different animals.

Conclusions

In this study, for the first time, an efficient and sensitive LC-MS-MS method was developed to detect, in buffalos, both the forms of bovine somatotropin included in the sustained release formulations available on the market.

Results obtained by analysing sera collected from buffalos treated for a 3-month period, according to a standard zootechnical protocol, allowed to understand the real conditions of applicability of the method and its effectiveness in terms of time window useful to identify treated animals. Such a window can be extended from the beginning of each cycle to approximately day 9, with some exceptions due to the great variability associated to the animal factor.

This significance of this study, beyond the development of a confirmation analysis protocol based on HPLC-MS-MS for detection of rbST in buffalos, is also to be related to the simulation of a real protocol of treatment involving several animals, which has never been presented before.

In conclusion, the method developed can be considered a valuable tool for control of illegal or undeclared hormone treatments of buffalos, providing unequivocal response on the presence of the recombinant forms of bovine somatotropin in serum samples.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and animal rights and informed consent Animal care and handling were performed according to the provisions of the relevant community legislation (Directive (EEC) n. 609/86; Directive 2010/63/EU). The experimental protocol has been approved by the Committee of the IZSLT (GR20091606346).

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