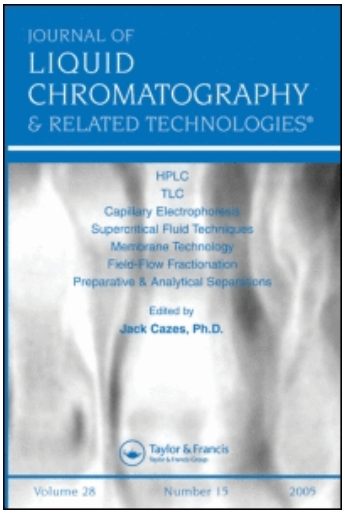

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EXTRACTION AND QUANTIFICATION OF RECOMBINANT BOVINE SOMATOTROPIN FROM OLEAGINOUS VEHICLE

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ABSTRACT

A non-denaturing assay for the determination of potency of a recombinant bovine somatotropin (somidobove) formulated in oleaginous vehicles was developed. In this paper, the optimal conditions for extraction of somidobove from oleaginous vehicle were investigated. Results demonstrated that somidobove monomer, oligomers, and soluble aggregates were quantitatively extracted by borate - ethylenediaminetetraacetate (EDTA) pH 9.5 buffer solution in a VanKel dissolution system at 40°C for 3 hours. The extracted analytes, somidobove monomer, dimer, and soluble aggregates, were separated and determined by using a high performance size exclusion chromatographic system consisting of a TSK G3000SW column and a borate - EDTA buffer (pH 7.3) mobile phase. This method was validated for the effects of buffer type, extraction temperature, extraction time and

stirring speed. The average CV of intra- and inter-day precision obtained in this method were 2.3%. The recovery of somidobove monomer was 93.7% with a coefficient of variance (CV) of 3.71%. The CV for precision generated in three different labs ranged from 1.3 - 3.9%. This method was linearly correlated with the hypophysectomized rat body weight gain assay, regression coefficient = 0.953.

INTRODUCTION

Somidobove is a recombinant bovine somatotropin (rbST) composed of 199 amino acids with a molecular weight 22,818. The difference between somidobove and native bovine somidotropin is that at the NH_2-terminus end of the protein, somidobove contains 9 additional amino acids. Somidobove has been developed for administration to dairy cows to increase milk yield and the efficiency of milk production for years. Several sustained release delivery systems[1,2,3] have been developed for somatotropin to eliminate the need for daily injection. These delivery systems yield sustained release of bioactive somidobove for periods of 7 to 28 days, are relatively inexpensive and easy to manufacture as sterile preparations, stable, and easy to administer to the animal. In this study, a mixture composed of sesame oil, white wax, propyl gallate was used as an oleaginous vehicle for the delivery of somidobove.

Many physico-chemical interactions, such as hydrophobic interactions, pH, salts, and denaturants, can lead to protein aggregation.[4,5] Proteins can also be denatured in the presence of gas-liquid interface.[6,7] The formation of soluble and insoluble aggregates in somatotropin formulations effects the release and pharmacokinetics of the somatotropin and reduces the potency and quality of the product. Developing analytical assays for the quantification of somatotropin potency in formulated products has presented challenges not only in accuracy (recovery) and precision, but particularly, the separation of non-denatured somatotropin and aggregates. The hypophysectomized (hypox) rat body weight gain assay is available for the determination of bulk somatotropin potency but not for somatotropin formulated in oleaginous vehicles due to the interference of the oleaginous excipients. Several detergents such as sodium dodecyl sulfate, have been used to extract somatotropin from oleaginous vehicles but the protein was strongly denatured in the extraction process. Additionally, non-covalent bonded oligomers and aggregates present in samples would be decomposed to the denatured monomer. As a consequence, excessively high results were obtained in these methods. The key point in the development of a somatotropin potency assay for formulated drugs is that the method is required to have a non-denaturing extraction procedure with

quantitative recovery. In order to retain the bioactivity of somatotropin in the extraction process, therefore, mild extraction conditions including weak alkaline buffer solution without any denaturants and a suitable low temperature were used for the extraction of somatotropin from oleaginous vehicle.

Authors have reported a non-denaturing size exclusion chromatographic (SEC) assay for the determination of somidobove potency in bulk drugs.[8,9] This method can not only be used to determine the potency of the somidobove monomer, but also to separate and estimate the oligomers and soluble aggregates present. In addition, this method has been correlated to the hypox rat body weight gain assay and can be used for the determination of somidobove potency in routine analysis.

This paper describes a non-denaturing extraction procedure and a size exclusion chromatographic method for the determination of somidobove potency in oleaginous vehicles. The optimization of the extraction procedure including the incubation time, temperature, stirring speed, and buffer solution was investigated. This method was validated and was linearly correlated with the hypox rat body weight gain assay.

EXPERIMENTAL

Materials

Recombinant bovine somatotropin reference standard, bulk somidobove, and formulated somidobove in oleaginous vehicle (sesame oil, white wax, propyl gallate) were provided by Eli Lilly and Company. Water used in this study was obtained from a Millipore Milli-Q water purification system. All reagents and chemicals were of analytical-reagent grade and were used without further purification. Three buffer solutions, 20mM sodium borate - 1.44mM EDTA, 25mM sodium monohydrogen phosphate, and 50mM ammonium bicarbonate, adjusted pH to 9.5 with NaOH were used as extraction solutions.

Extraction

A Vankel Industries Vanderkamp 6000 six-spindle dissolution tester equipped with stir paddles and 1000 mL dissolution vessels was used for the extraction. The temperature of extraction solutions was controlled by a Vankel

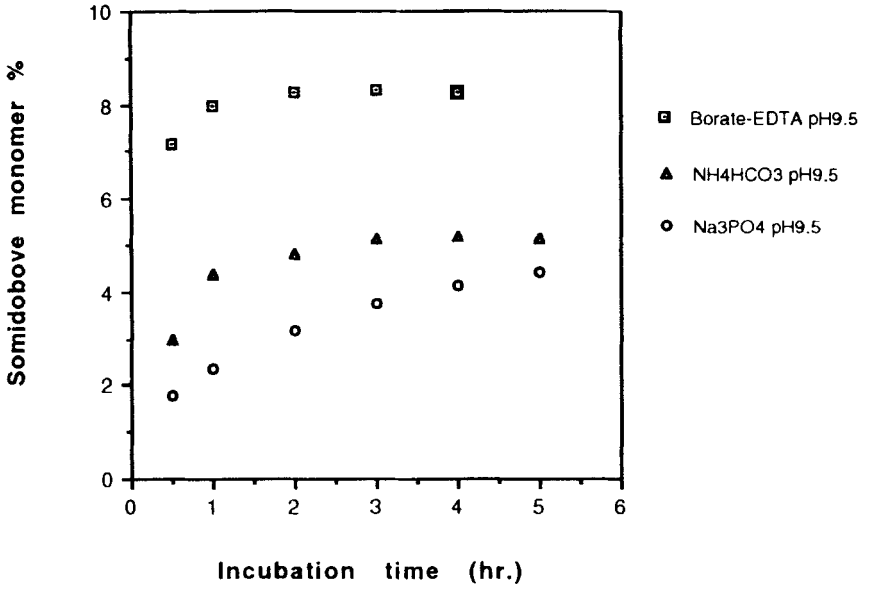

Figure 1. Extraction of somidobove from oleaginous vehicle by different buffer solutions. Extraction conditions: Formulated drug sample (Lot OA18); stirring speed 100 ± 4 rpm; incubation temperature was controlled at ± 0.2° C. Chromatography was performed on a TSK G3000SW column with a mobile phase of 20mM borate - 1.44mM EDTA buffer pH 7.3 at 0.5 mL/min flow rate. Elutes were detected at 280 nm.

C-2600 Heater/Circulator. Two to four grams of the formulated drug sample, depending upon the content of somidobove in the sample was accurately (±0.001g) weighed and transferred into the bottom of each dissolution vessel. 500 mL of the extraction buffer solution was added into each of the vessels. The vessels were placed into the dissolution apparatus with a temperature of 40 ± 0.5°C. Paddles were then lowered into the extraction solution until the top of each stir paddle was 5 mm below the surface of solution. The dissolution apparatus was set up to stir at 100 ±4 rpm. The covers were placed on each vessel and paddles were started in motion.

The incubation was maintained for 3 hours. After completion of the extraction, spindle motion was stopped. An aliquot of the extracted solution was removed by inserting a pipette into the solution placed within 1.5 - 2 inches from the vessel bottom.

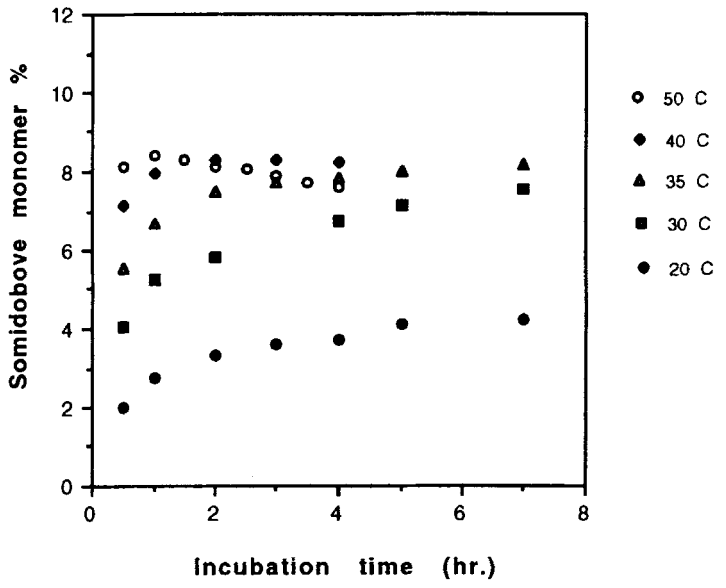

Figure 2. Effect of incubation time and temperature on the recovery of somidobove in extraction. Experimental conditions were the same as in Figure 1 except the extraction solution: 20 mM Na borate - 1.44 mM EDTA, pH 9.5.

Prior to sampling, a small amount of air was expelled from the pipette tip to remove any vehicle which may have been trapped in the pipette tip while inserting the pipette into the solution. The sample solution was filtered through a 0.45 μm Acrodisc filter and analyzed by HPLC.

Chromatography

HPSEC was performed on a HPLC system consisting of a Waters 625 LC system with a 991+ photodiode array detector and WISP 712 autosampler (Waters Chromatography, Milford, MA, USA). Varian 5000 Liquid Chromatograph (Varian Instruments, San Fernando, CA, USA) with a Spectroflow 757 absorbance detector (Kratos Analytical Inc., Ramsey, NY, USA) was used for the study. TSK G3000SW columns, 7.5x300mm, were operated at ambient temperature and with a 20 mL injection volume. The mobile phase was 20mM sodium borate - 1.44 mM ethylenediaminetetraacetate di-sodium salt (EDTA) buffer solution adjusted to pH 7.3 with hydrochloric acid.

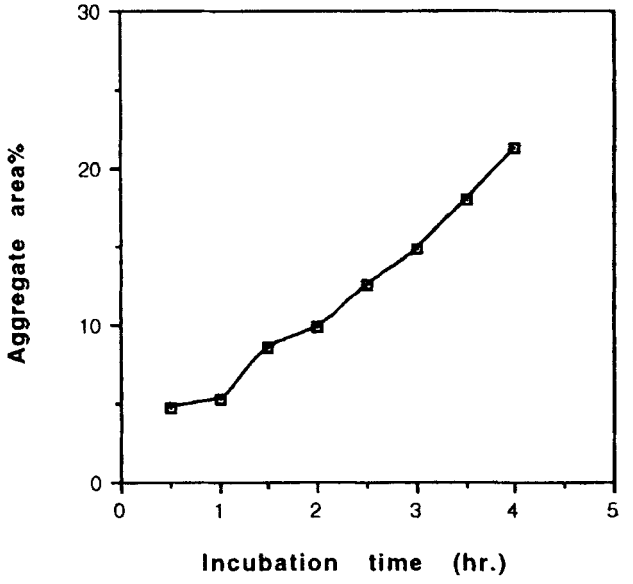

Figure 3. Effect of the incubation time on the formation of somidobove soluble aggregates at 50°C. Experimental conditions were the same as in Figure 2 except the incubation temperature at 50°C.

A flow rate of 0.5 mL/min was used for most of the studies in this work. Eluted components were detected at 280 nm. A linear regression plot of the reference standard in mg/mL versus peak area was used for the quantification of somidobove monomer and peak area normalization was employed for the estimation of the dimer and aggregates in formulated drug samples. A HP-1000 computer system (Hewlett - Packard, San Fernando, CA, USA) was used to collect, store and analyze the chromatographic data.

RESULTS AND DISCUSSION

Extraction

Somidobove maintains bioactivity in pH 9.5 borate-EDTA.[7,8] As a first choice, this buffer was used to extract somidobove from oleaginous vehicles. Several extraction procedures were investigated for the recovery of somidobove in the extraction process. When somidobove bulk drug was shaken with a

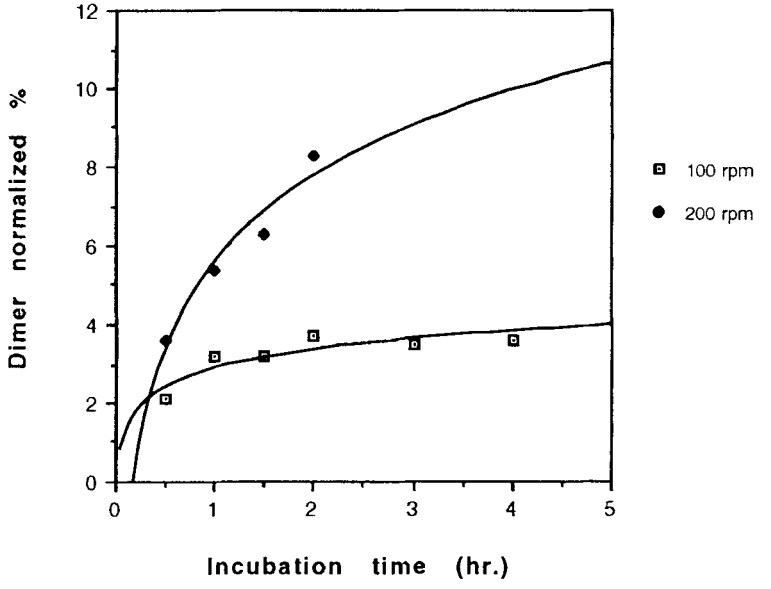

Figure 4. Influence of stirring speed in extraction on the formation of somidobove dimer. The experimental conditions were the same as in Figure 1.

borate - EDTA buffer solution heated at 40 - 50° C, the rate of protein denaturation increased resulting in the formation of both soluble and insoluble protein aggregates due to solid-liquid interfacial effect. Somidobove aggregates can also be formed in solution by strong stirring. This leads to poor sample recovery. In order to prevent the aggregation of protein in solution, mild conditions such as low incubation temperature, slow constant stirring, and weak alkaline bufer were employed in the extraction process.

Three different extraction solutions, 20mM borate - 1.4mM EDTA (pH 9.5), 25mM phosphate buffer (pH 9.5), and 50mM ammonium bicarbonate (pH 9.5) were evaluated for the extraction efficiency of somidobove from oleaginous vehicle. Figure 1 shows the influence of different buffer solutions on the recovery of somidobove monomer from oleaginous vehicle. Both phosphate and bicarbonate buffers showed poor extraction recovery for somidobove monomer. About 50-60% extraction recovery was found by using both buffers after 5 hours incubation. However, the borate-EDTA buffer provided excellent extraction recovery and stability. The somidobove extraction was quantitatively completed within 2 - 3 hours by using this buffer. The incubation temperature significantly affected the extraction recovery of somidobove from oleaginous

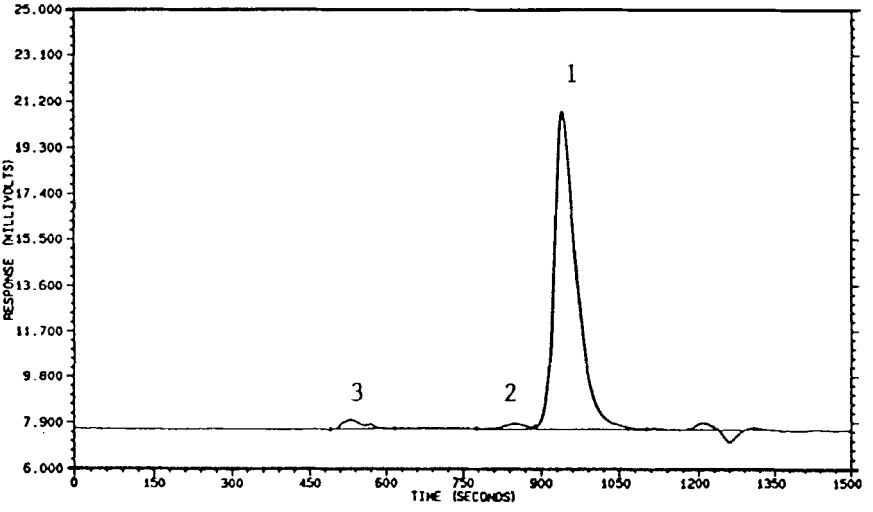

Figure 5. HPSEC chromatogram of somidobove extracted from the oleaginous vehicle lot 024. Peak 1: rbST monomer; Peak 2: Dimer; Peak 3: Soluble aggregates. Extraction and chromatographic conditions were the same as in Figure 1 except the incubation temperature was controlled at 40°C.

vehicles. It was found (Figure 2) that a low incubation temperature led to a lower recovery of somidobove even when the incubation time was prolonged to 7 hours in the extraction process. About 50% recovery was obtained in the extraction solution when incubated at 23°C and about 90% recovery was obtained at 30°C after 7 hours.

The formulated sample incubated at 35°C needed 6 hours to complete the extraction. Although a higher incubation temperature (50°C) reduced the incubation time, the somidobove potency was lost because more aggregates are formed in the extraction process (Figure 3). The results in this study revealed that the monomeric somidobove can be quantitatively extracted by using a borate-EDTA, pH 9.5, buffer solution at 40°C within 2-3 hours. Under these mild conditions, even if the incubation time was prolonged to 5 hours, the somidobove potency would be maintained.

Owing to the significant influence of strong shaking and stirring on the recovery in extraction, the optimal condition in stirring speed was investigated. A high stirring speed (200 rpm) caused a significant increase of somidobove

Table 1

Precision of Somidobove Monomer Determined in Formulated Drug Lots

Sample	Somidobove Monomer							
	Day 1		Day 2		Day 3		Mean	
Lot	%	CV%	%	CV%	%	CV%	%	CV%
024 (n=6)	8.37	1.56	8.40	1.18	8.65	1.94	8.47	1.75
4003A (n=6)	9.75	2.25	9.44	0.94	9.73	1.23	9.64	1.80
0A18 (n=6)	16.33	3.17	17.21	3.21	17.02	1.21	16.85	2.75

dimer (Figure 4). Overly slow stirring speed led to incomplete extraction and poor recovery. A stirring speed with 100 ± rpm revealed the optimal conditions for quantitative extraction of somidobove from oleanginous vehicle.

Quantification

Figure 5 shows a typical SEC chromatogram of somidobove extracted from oleaginous vehicle. Under the conditions described, the monomeric somidobove is baseline separated from dimer and soluble aggregates. The elution time of somidobove monomer is 15.6 min.

Linearity and Limit of Quantitation

The peak area response were linearly related to the concentration of somidobove in formulated vehicle in the range from 0.09 - 0.93 mg/mL. The regression equation was y (peak area response) = 4938.0 x (somidobove mg/mL) - 87.6. Correlation coefficient of the linearity was 0.999. The limit of quantitation (LOQ) in this method was 0.15mg/mL of somidobove monomer.

Precision and Reproducibility

Method precision for the determination of somidobove monomer potency in oleaginous vehicles was assessed through six weighings of three different formulated lots on three separate days. Table 1 summarizes the intra-day and

Table 2

Ruggedness of Precision and Recovery

Somidobove Monomer %

	Analyst 1	Analyst 2	Analyst 3	Inter-Lab
Lot 024				
Mean(n=3)	8.42	8.08	8.74	8.41
CV%	1.68	3.29	0.91	3.92
Lot 4003A				
Mean(n=3)	9.50	9.25	9.91	9.55
CV%	0.58	0.97	0.56	3.49
Lot 0A18				
Mean(n=3)	16.86		17.17	17.02
CV%	2.33		1.05	1.29
Lot 4004A				
Mean(n=3)	9.36	9.12	9.66	9.38
CV%	1.45	0.70	2.60	2.88

inter-day precision data. The coefficient of variance (CV) of intra-day precision in this study was 0.9 - 3.3% and CV of inter-day precision was 1.8-2.8%. System reproducibility was determined by injecting 5 times the same extracted solution of lot 0A18. The CV obtained in this experiment was 2.6%.

Accuracy (Recovery)

The method accuracy was assessed by fortifying two different amounts of somidobove bulk drug lot into a formulated drug lot 024 and analyzed by this method. The average recovery obtained was 93.7% with a CV of 3.71%.

Stability

The stability of somidobove in extraction solutions was evaluated by using two sets of identical sample solutions at room (22°C) and refrigerator (2-8°C). Results indicated that under the both temperature conditions, extracted monomeric somidobove in solution was stable for 2 days. The average CV was <1.6%.

Ruggedness

The ruggedness study was carried out by three analysts using 4 different formulated drug lots on different instruments at three different labs. The data of ruggedness were summarized in Table 2. The CV of precision generated at three different labs ranged from 1.3 - 3.9%.

Correlation with Hypox Rat Body Weight Gain Assay

The correlation between the potency data obtained by both of hypox rat body weight gain assay and this method was studied. Different amounts of somidobove formulated drug samples were extracted and assayed by this method. The extracted solutions with different concentrations of somidobove were then measured by hypox rat assay. The data obtained by both assays was linearly correlated. The regression coefficient obtained was 0.953.

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