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Journal of Chromatography A, 675 (1994) 113–122

JOURNAL OF CHROMATOGRAPHY A

Non-denaturing assay for the determination of the potency of recombinant bovine somatotropin by high-performance size-exclusion chromatography

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(First received February 18th, 1993; revised manuscript received March 18th, 1994)

Abstract

A non-denaturing high-performance size-exclusion chromatographic method has been developed for the determination of the potency of recombinant bovine somatotropin (rbST) in bulk materials. A Spherogel TSK 3000 PW column containing a polymer base packing material with very hydrophilic bonded surface was used in this method. Ammonium hydrogencarbonate buffer pH 9.0, was used as the mobile phase. This method was shown to be non-denaturing by rat mass-gain assay and radioreceptor assay. The optimization for the separation and determination of rbST has been investigated. The method was validated for the determination of rbST in bulk materials.

In addition, rbST soluble aggregates formed in the production process due to protein association, used to be found in bulk materials. The behavior of rbST soluble aggregates in ammonium hydrogencarbonate solutions have been studied. The bio-inactive aggregates can be separated by the method developed in this study. The high–low chromatographic technique has been used to estimate rbST soluble aggregates in bulk materials.

1. Introduction

Recombinant DNA-derived bovine somatotropin (rbST) is under development for increased milk production and improvement of efficiency of production in lactating dairy cows. It is important to establish an assay for the determination of the potency of rbST for quality control. Several methods for determination of human growth hormone have been reported [1–5] but only a few publications for the quantification of rbST were found in the literature [6–8]. For years, the potency of rbST has been estimated by a rat mass-gain method [9]. Recently, a radioreceptor assay has been developed for the biopotency of rbST [10]. This method appears to be more accurate than the rat mass-gain method. However, these assays are time-consuming and less reproducible to be used to quantify rbST in routine analysis. In this case, it could be considered to determine the potency by physico-chemical techniques. In recent years, reversed-phase high-performance liquid chromatography (RP-HPLC) has been employed for rbST determination [7,8]. Nevertheless, RP-HPLC was not an appropriate method for the determination of rbST potency because of the denaturation and degradation produced in the chromatographic

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0021-9673/94/$07.00
SSDI 0021-9673(94)00281-D

process by acidic medium and organic solvents used in the mobile phase. The results obtained in the study of the effect of acidification indicated that bovine growth hormone results in increased unfolding of the hormone as the pH in solution was reduced from 5 to 2 [11]). High-performance size-exclusion chromatography (HPSEC) has been employed to assay rbST [6–8] but these methods cannot be used as potency assays because of the denaturation in mobile phases containing sodium dodecyl sulfate (SDS) or 6 *M* guanidine hydrochloride (Gn · HCl), in the methods.

rbST is composed of 199 amino acids [12] with a molecular mass of 22 818. rbST shows a stronger hydrophobicity (calculated value 478) [13] because of 69 hydrophobic amino acids including 28 leucine, 14 alanine, 14 phenylalanine and 7 isoleucines in the molecule. Selection of a stationary phase in SEC for this protein was difficult. The packing materials used in SEC for rbST potency determination had to be very hydrophilic. Consequently, the strong hydrophobic protein rbST would not be adsorbed on the packing material surface in the SEC process. Most HPLC packing materials are made of silica gel as a supporting base and can only be used at pH lower than 7.4. rbST maintains bioactivity in weak alkaline solution. Therefore, polymer-base packing materials with hydrophilic bonded surface would be available for the separation and determination of rbST potency.

Based upon the requirements mentioned above, a non-denaturing HPSEC assay was developed in this study for the determination of rbST potency in bulk materials. Spherogel TSK 3000 PW column containing a polymer-base packing material with very hydrophilic bonded surface was applied in this method with ammonium hydrogencarbonate buffer used as a mobile phase. This method was shown to be non-denaturing by rat mass-gain assay and radioreceptor assay. The optimal conditions which include control of salt concentration, pH in the mobile phases and flow-rate, have been investigated for the separation and determination of rbST. This method has been validated for the determination of rbST in bulk materials.

rbST soluble aggregates formed in the production process due to protein association, used to be found in bulk materials. These aggregates are bio-inactive. Therefore, it is important to develop a method to determine rbST soluble aggregates for quality control.

In pharmaceutical analysis, often a reference standard is not available for each component, mostly only for main components. In this case, an alternative method was required to use for the quantitation of impurities in samples. High–low chromatography is a technique that can be employed to estimate trace impurities in samples [14]. Generally, the high–low approach utilizes a pair of sample solutions with a higher concentration and a lower concentration. In the chromatogram obtained from a higher-concentration sample solution, the main component response is off-scale and the impurity peak response is in the linear range of the detection system. The main component peak response in the solution with a lower-concentration sample concentration, 1 to 50 or 100 dilution of the higher-concentration sample solution, was quantitated in the linear region of the detection system. The percentage of the impurity can be calculated by comparing the peak responses in the pair of chromatograms because the concentration difference between the two solutions is known. The limitation of this approach is that it requires close peak absorptivity between the main component and the impurity if UV–visible detection is used. In addition, the selection of chromatographic conditions is important to provide accurate results.

In this study, the high–low chromatographic technique was used to estimate soluble aggregates in rbST bulk materials. Behavior of rbST soluble aggregates in solutions has also been investigated.

2. Experimental

2.1. Chemicals and reagents

All reagents were of analytical-reagent grade and were used without further purification. Water was obtained from a Millipore Milli-Q water purification system. SDS (Aldrich, Mil-

waukee, WI, USA) and Gn·HCl (Sigma, St. Louis, MO, USA) were used as denaturing agents. Protein standards were obtained from Sigma molecular mass marker kits. The rbST reference standard and bulk materials were obtained from Eli Lilly and Company (Indianapolis, IN, USA).

2.2. Chromatography

The majority of the study was performed with a Waters 625 LC system with a 991+ photodiode array detector and a WISP 712 autosampler (Waters Chromatography, Milford, MA, USA). The validation work was carried out on a Beckman System Gold HPLC system (Fullerton, CA, USA) consisting of a Model 126 solvent delivery system, a Model 166 variable-wavelength detector, and a Model 507 autosampler. All measurements were monitored at 280 nm. Spherogel TSK 3000 PW columns (300 × 7.5 mm I.D.) (Beckman, Palo Alto, CA, USA) and TSK gel G2500PWXL column (300 × 7.8 mm I.D.) (TosoHaas, Montgomeryville, PA, USA) were employed for most of the studies except as indicated. The mobile phase was 0.2 *M* ammonium hydrogencarbonate adjusted to pH 9 with NaOH. Ammonium hydrogencarbonate 0.2 *M* (pH 9) with 1% SDS was used as a mobile phase in the SDS SEC study. All separations were achieved at room temperature with a flow-rate of 0.5 ml/min. The injection volume was 20 μl. The quantity of rbST present in the samples was calculated by constructing a linear regression plot of concentration of rbST in mg/ml *versus* peak area. All chromatographic data were collected, stored and analyzed on a HP-1000 computer system (Hewlett-Packard, San Fernando, CA, USA).

2.3. Sample and reference standard preparation

Samples were typically prepared at a concentration of 0.5–0.8 mg/ml in the mobile phase. To minimize incomplete dissolution, an aliquot of mobile phase was added and the sample solution was allowed to stand at room temperature for 30 min. After that the solution was gently shaken for 5–10 min and then diluted to volume. To remove insoluble substances, sample solutions were filtered through an acrodisc low protein binding filter (0.45 μm) prior to chromatography. rbST reference standards were immediately prepared in mobile phase at three concentrations ranging between 0.1–1.0 mg/ml.

2.4. High–low chromatography

In high–low chromatography, a 10 mg/ml solution of rbST sample was prepared in the mobile phase and filtered through an Acrodisc filter (0.45 μm) as a high-concentration sample solution. A low-concentration sample solution was obtained by making a 1 to 50 dilution of the high-concentration sample solution with the mobile phase. Both solutions were chromatographed under identical experimental conditions. The percentage of minor components (soluble aggregates) can be calculated as

$$\% \text{ Soluble aggregates} = (A_{SA}/50)/[A_M + (A_{SA}/50)] \cdot 100$$

where A_{SA} is the peak response of the soluble aggregates that was measured from the high-concentration chromatogram and A_M is the peak response of rbST main component from the low-concentration chromatogram.

3. Results and discussion

3.1. Separation

Fig. 1 shows chromatograms of three rbST lots that were obtained on a Spherogel TSK 3000PW column with 0.2 *M* ammonium hydrogencarbonate (pH 9.0) mobile phase. In the chromatograms, a main peak at 840 s in all three samples was identified as the rbST monomer. Peaks eluting around 590 s, the total exclusion volume of the column, in chromatograms B and C may be rbST soluble aggregates with very high molecular mass. No aggregates were found in lot 001 but lot 003 contains higher than 30% soluble aggregates, and lot 002 had approximately 7%

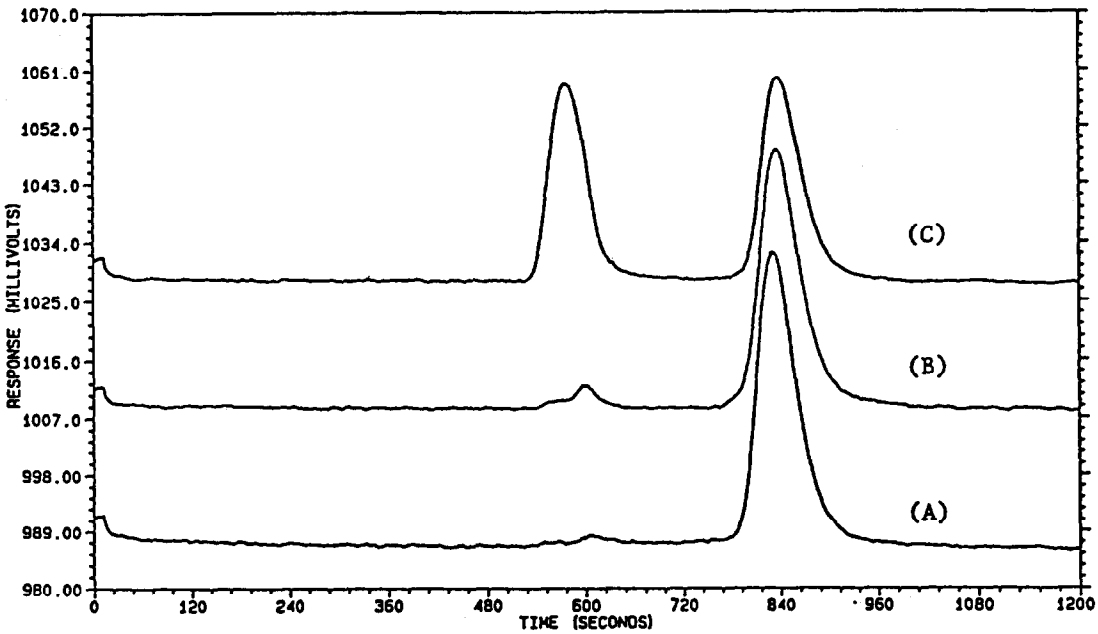

Fig. 1. SEC chromatograms of rbST sample lots. Column: TSK Spherogel 3000 PW (300 × 7.5 mm). Mobile phase: 0.2 *M* ammonium hydrogencarbonate (pH 9) in water. Flow-rate: 0.5 ml/min. Sample concentration: 1 mg/ml. Injection volume: 20 μl. (A) lot 001; (B) lot 002; (C) lot 003.

soluble aggregates. In order to obtain high column efficiency in the separation, a TSK 2500PWXL column packed with 5-μm polymer-base packing material had been tried to use for the separation of rbST. The poor resolution between rbST soluble aggregates and monomer, that is due to smaller permeation volume of this packing material, was found even though a sharper peak of the rbST monomer was obtained on this column.

3.2. Molecular mass measurement

The apparent molecular masses of rbST monomer and soluble aggregates were determined in 0.1 *M* NH_4HCO_3–0.1 *M* NaCl solution based upon a standard protein calibration curve (Fig. 2, plot A). Using several lots, the molecular mass of the rbST monomer was found to be 23 800. The molecular mass of the soluble aggregates was determined to be larger than 400 000 because it eluted at the total exclusion volume of the column. The molecular mass of rbST monomer was also examined in a mobile phase containing denaturing agents such as SDS and Gn · HCl. The higher molecular mass was obtained in a mobile phase containing SDS ($M_r \approx 25\,000$) and Gn · HCl (34 000). This may be due to unfolding of the protein [15,16] and an increase in molecular size of the SDS–protein complex.

3.3. Optimal HPLC conditions

The effect of ammonium hydrogencarbonate concentration on the elution time of rbST was investigated. A typical plot of elution time (t_e) *versus* buffer concentration [NH_4HCO_3] is shown in Fig. 3, plot A. A slight change in elution time was observed as the ammonium hydrogencarbonate concentration ranged from 0.1 to 0.4 *M*. Elution time increased more rapidly above 0.4 *M* ammonium hydrogencarbonate. The peak area of rbST monomer did not change in the concentration of ammonium hydrogencarbonate below 0.7 *M*, but dramatically decreased when increasing the salt

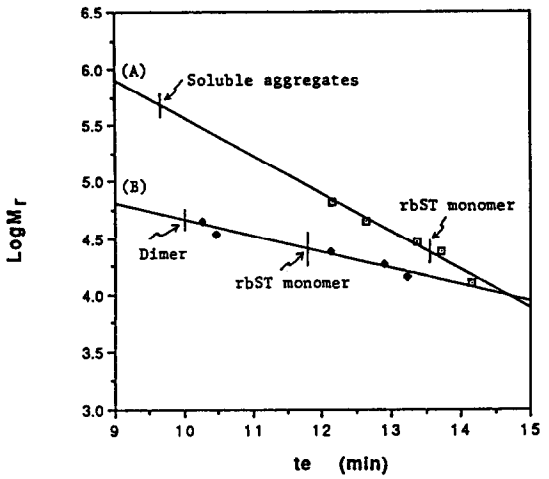

Fig. 2. Molecular mass calibration plots for TSK Spherogel 3000 PW column. Plot A: Mobile phase is 0.1 *M* ammonium hydrogencarbonate–0.1 *M* NaCl (pH 9.0) solution. *y*-Intercept = 8.94; slope = 0.337; correlation coefficient = 0.961. Protein standards: bovine serum albumin (M_r 66 000); ovalbumin (M_r 45 000); carbonic anhydrase (M_r 29 000); trypsinogen (M_r 24 000); cytochrome *c* (M_r 12 400). Plot B: Mobile phase is 1% SDS in 0.2 *M* ammonium hydrogencarbonate solution (pH 9.0). *y*-Intercept = 6.10; slope = 0.144; correlation coefficient = 0.959. Protein standards: ovalbumin (M_r 45 000); pepsin (M_r 34 700); trypsinogen (M_r 24 000); β-lactoglobulin (M_r 18 400); lysozyme (M_r 14 300). Flow-rate = 0.5 ml/min. The concentration of protein standards is 1 mg/ml in water. Injection volume is 20 μl.

concentration to higher than 0.7 *M*. This was due to the salting-out effect of the protein (Fig. 4, plot A).

A 0.2 *M* ammonium hydrogencarbonate solution was selected as a mobile phase for the study of pH effect. When increasing the pH in the mobile phase, the elution time of the rbST monomer remains constant but the peak area was slightly increased (Fig. 5, plot A).

The behavior of soluble aggregates in hydrogencarbonate solution has also been investigated. The solubility of rbST soluble aggregates was strongly effected by pH and hydrogencarbonate concentration in solution. Increasing hydrogencarbonate concentration to higher than 0.1 *M* caused the solubility of soluble aggregates to sharply decrease because of the strong salting-out effect of the huge molecules (Fig. 4, plot B). Soluble aggregates precipitate in the 0.2 *M* hydrogencarbonate solution at pH below 8.5.

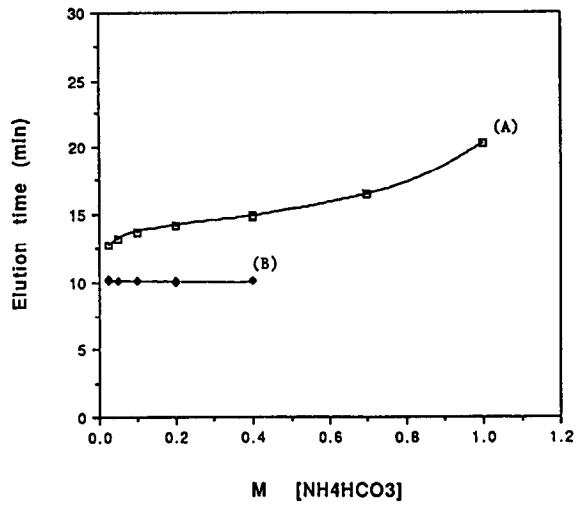

Fig. 3. Effect of hydrogencarbonate concentration on retention times. Same conditions as in Fig. 1 except the concentration of bicarbonate in the mobile phase changed from 0.025 to 1 *M*. The rbST sample used in this experiment is lot 003. Plot A: rbST monomer; plot B: rbST soluble aggregates.

The solubility increases with increasing pH to 9.5 and then remains no significant change until pH 10.5 (Fig. 5, plot B). These results demonstrated that rbST soluble aggregates can be removed in buffer solutions with pH below 8. No significant influence of pH and salt concentration on elution

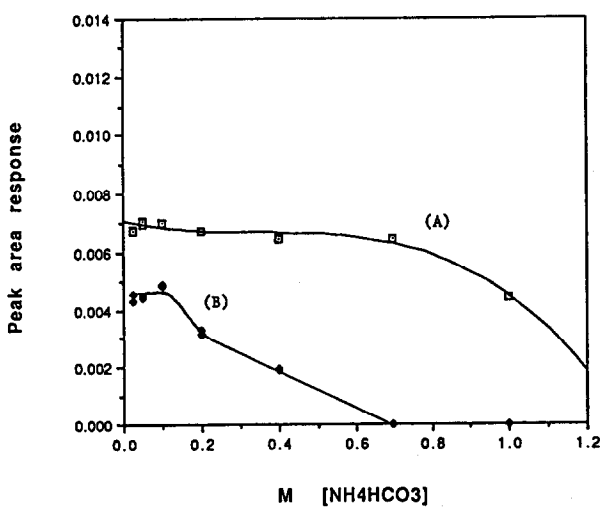

Fig. 4. Effect of hydrogencarbonate concentration on peak areas. Same conditions as in Fig. 3. Plot A: rbST monomer; plot B: rbST soluble aggregates.

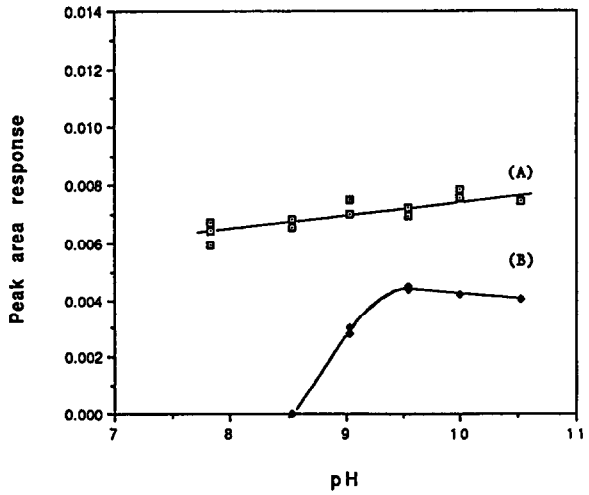

Fig. 5. Effect of pH variation in the mobile phase on the peak area of bST. Same condition as in Fig. 1. Plot A: rbST monomer; plot B: rbST aggregates.

time of the soluble aggregates was found in this study

The optimal flow-rate in this method was 0.5 ml/min. A flow-rate of 0.3 ml/min results in equivalent efficiency but nearly doubles the run time. A higher flow-rate of 1.0 ml/min results in the rbST main peak being partially overlapped with the void peak.

3.4. Non-denaturation

The non-denaturation of the rbST monomer in the separation has been confirmed by rat mass-gain assay and radioreceptor assay. Two different lots, 003 and 004, were used in this study. Lot 004 contained less than 1% aggregates, but more than 30% of soluble aggregates were present in lot 003. The rbST monomer in both lots was semi-preparatively separated and collected under the same experimental conditions as described above. The bioactivity of the monomer fractions collected from both lots was measured by the rat mass-gain assay and found to be close to that of the reference standard (Table 1). The soluble aggregates fraction collected from lot 003 showed no bioactivity by the rat mass-gain assay.

Results obtained in the radioreceptor assay (RRA) for the lot 003 and the reference standard were consistent with that in rat mass-gain assay. It indicated that the bioactivity of rbST monomer collected in both samples remains identical but the soluble aggregates collected from lot 003 showed no bioactivity using the radioreceptor assay (Table 1). A linear correlation was found between chromatography and radioreceptor data in different lots of bulk materials. A relatively poor correlation coefficient of 0.845 was obtained due to the limitation of the reproducibility of the RRA method.

The results obtained in this study clearly indicated that the separation conditions in this study can be applied to establish a non-denaturing potency assay for determination of rbST in bulk materials.

3.5. Soluble aggregates and dimer

The formation of rbST aggregates during the production process has been recognized. In order to identify the soluble aggregates, a num-

Table 1
Bioactivity confirmation by rat mass-gain assay (RMGA) and radioreceptor assay (RRA)

	Potency (IU/mg) RMGA			RRA	
	RS	Lot 004	Lot 003	RS	Lot 003
rbST Main component					
Original solution	1.25	1.04		1.2	1.1
Collected in SEC		1.29	1.19	1.2	1.1
rbST aggregates collected			0		0

RS = Reference standard.

ber of experiments were carried out in this study. The UV spectrum provided by on-line photodiode array detector in the chromatographic process of lot 003 demonstrated that the UV profiles of both rbST monomer and soluble aggregates are very similar. A slight differentiation at 250–270 nm (Fig. 6) for both proteins may be due to the change in environment of the aromatic amino acids in the molecule after aggregation.

The stability of rbST soluble aggregates in hydrogencarbonate solution has been investigated. The isolated soluble aggregates are relatively stable in pH > 9 ammonium hydrogencarbonate solution at least for 9 h, particularly at low concentration.

Denaturing agents, such as detergents, Gn · HCl and urea, decompose rbST aggregates into denatured monomer [15–17]. As 1% SDS was added to the mobile phase, the aggregate peaks in lot 002 and 003 disappeared in the chromatograms as shown in Fig. 7. rbST monomer peak was broadened and a small peak was found in front of the main peak that may be a covalent bond dimer of rbST. Similar results were obtained in a 0.2 *M* NH_4HCO_3, pH 9 mobile phase containing 6 *M* Gn · HCl.

To confirm the above presumption, the soluble aggregate fraction was collected from lot 003 in the system consisting of a TSK 3000 PW column and 0.2 *M* ammonium hydrogencarbonate mobile phase. The collected fraction was mixed 1:1 with 2% SDS solution and re-chromatographed on the same column with a 1% SDS–0.2 *M* NH_4HCO_3 mobile phase. An identical profile and elution time were obtained as the SDS-monomer peak The same results were obtained in the mobile phase with Gn · HCl. These results illustrated that in the presence of denaturing agents in solutions, rbST soluble aggregates (non-covalent bonded) would be decomposed into denaturated monomer. It is important to realize that if rbST soluble aggregates existed in bulk material, an excessively high result would

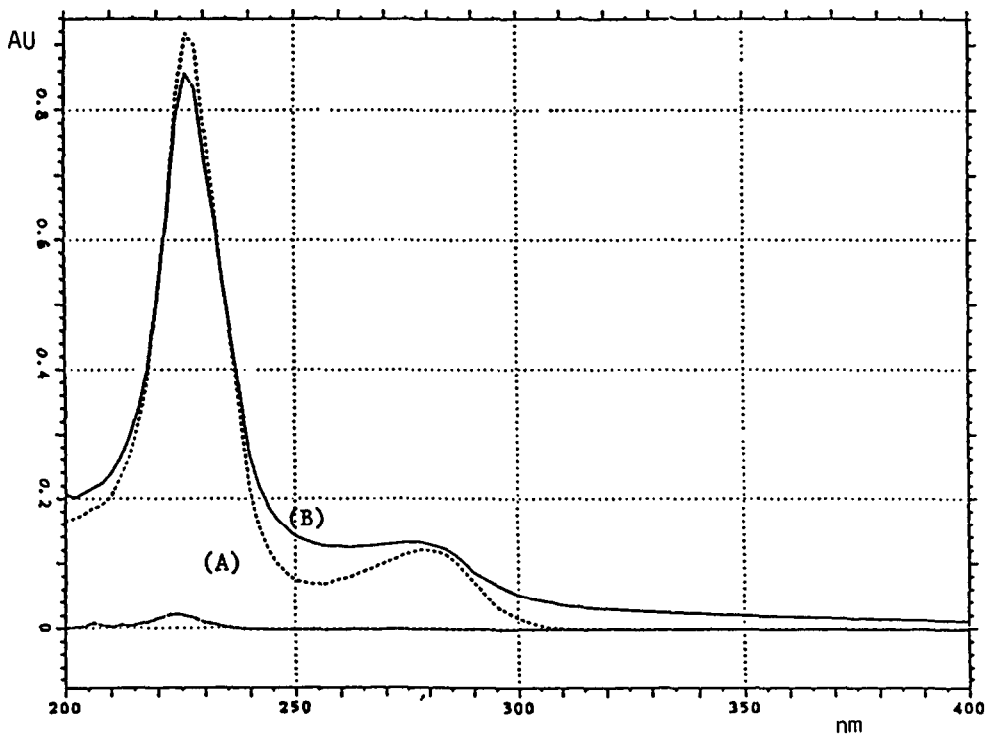

Fig. 6. HPSEC on-line UV spectrum of rbST monomer and soluble aggregates. (A) rbST monomer, (B) rbST soluble aggregates. Chromatographic conditions as in Fig. 1.

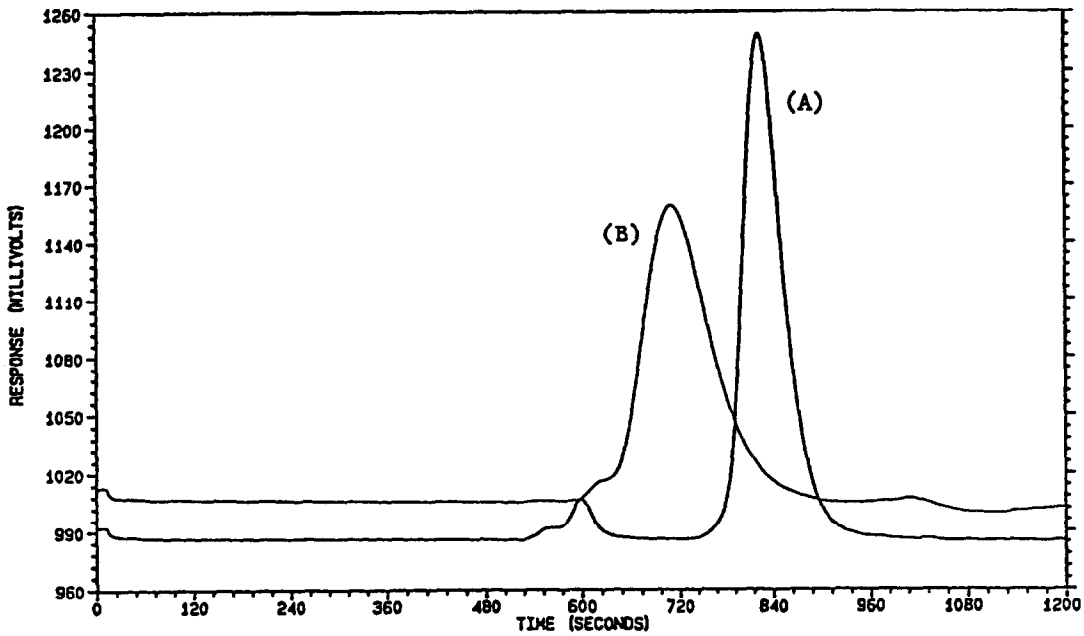

Fig. 7. SDS SEC chromatograms of lot 002 bulk material. Column: TSK Spherogel 3000 PW (300 × 7.5mm). (A) In 0.2 *M* ammonium hydrogencarbonate (pH 9.0); (B) in the mobile phase A with 1% SDS. Sample concentration: 1 mg/ml; flow-rate: 0.5 ml/min.

be obtained in quantitative work by these denaturing analytical procedure [6–8] because of the decomposition of aggregates.

The above results also indicated that the covalent bonded dimer of rbST may be present in rbST lots. The rbST covalent bonded dimer, mostly in low concentration ($<3\%$), cannot be completely resolved in the system of a TSK 3000 PW column and pH 9 ammonium hydrogencarbonate because of the limitation of the less efficiency of polymer packing materials. In the presence of denaturing agents such as SDS and Gn · HCl in the mobile phase, however, the resolution increased and covalent bonded dimer could be separated from the rbST monomer (Fig. 7). It provides a possibility to estimate rbST dimer by using the high–low chromatographic technique or normalization procedure. The molecular mass of covalent bonded dimer in SDS–NH_4HCO_3 mobile phase has been estimated to be 46 000 by utilizing standard protein calibration curve as shown in Fig. 3. The further study for the characterization of rbST dimer has been carrying out in our laboratory,

3.6. Quantification and validation for rbST monomer

Quantification of rbST monomer was achieved using an external standard for the determination of rbST monomer potency. The linearity of rbST monomer in 0.2 *M* ammonium hydrogencarbonate mobile phase was measured by preparing a number of rbST reference standard solutions with different concentrations. The linear regression analysis was performed in the concentration range from 0.1 to 1 mg/ml. The correlation coefficient ranged from 0.9998 to 1.000 and the average relative standard deviation (R.S.D.) was 1.36%.

The precision of this method was evaluated by three days using two different chromatography systems, three different columns and three different lots of rbST bulk materials. The average

Table 2
Estimation of rbST soluble aggregates by high–low chromatography

Sample lot	Soluble aggregates (%)	R.S.D. (%)
002	8.8	4.6
008	0.76	5.0

R.S.D. of rbST monomer measured was less than 2.8% and the reproducibility of elution time was less than 0.18%.

The recovery study for rbST monomer was carried out by addition of reference standard at two different concentrations in the bulk material solutions. The recoveries obtained in this experiment ranged from 101 to 104%.

The stability of the rbST monomer in solution was measured at room temperature by using three rbST reference standard solutions at different concentrations. The rbST content in freshly prepared solutions was determined and then compared with the data obtained from an aged solutions which were stored at room temperature for 30 h. Data obtained in this experiment indicated that no significant change in results was found within 30 h.

3.7. Estimation of soluble aggregates

A single sample high–low procedure described in the Experimental section with a dilution ratio of 50 was used in this study. Good correlation was found between peak response of soluble aggregates and concentration in the range from 0.014 to 0.362 mg/ml. The results obtained for two rbST lots are listed in Table 2.

4. Conclusions

(1) The non-denaturing HPSEC assay developed in this paper provides for the accurate and precise determination of rbST potency in bulk materials. The non-denaturation of this method was confirmed by rat mass-gain assay and RRA.

(2) The rbST soluble aggregates can be resolved from the monomer in this method and estimated by the high–low chromatographic technique.

(3) The experimental results demonstrate that in the presence of denaturing agents such as SDS and Gn · HCl, non-covalent bonded aggregates dissociate to rbST. Thus, an increase in rbST monomer content is obtained when denaturing mobile phases are used in the assay.

Acknowledgements

The authors thank Dr. Bruce H. Frank, Dr. Ralph M. Riggin and Dr. Gregory F. Needham for very useful discussions; Dr. Eugene L. Inman, Mr. J. Matt Rodewald and Mr. Daniel J. Sweeney for reviewing the manuscript and Mr. Paul A. Record for the rat mass-gain assay.

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