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Journal of Chromatography A, 709 (1995) 249–256

JOURNAL OF CHROMATOGRAPHY A

Development of an analytical reversed-phase high-performance liquid chromatography assay for transforming growth factor β3

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First received 24 March 1995; revised manuscript received 10 April 1995; accepted 10 April 1995

Abstract

Transforming growth factor β3 (TGF-β3) is a dimeric protein (M_r 25 444) which regulates the proliferation of epithelial cells. A reversed-phase high-performance liquid chromatography (RP-HPLC) method was developed for TGF-β3 in order to investigate the solution stability of TGF-β3. Peak width and retention time were evaluated as a function of (1) the initial percent of acetonitrile, (2) the gradient slope, (3) the percentage of trifluoracetic acid, and (4) column temperature. By varying the above conditions, particularly temperature, it was possible to reduce retention times and peak widths by a factor of 2 and 6, respectively. The observed temperature effects on peak width were consistent with the concept that conformational changes can be induced in the protein during the chromatographic migration. A balance was found between on-column degradation and improved peak width at elevated temperatures. The final assay was found to be linear over a 10–150 μg/ml drug range with a correlation coefficient of 0.998 and acceptable reproducibility (R.S.D. 2.09%, $n = 6$). The assay has been useful for monitoring drug loss in ongoing stability studies; however, it was found to have poor resolving power when certain stressed samples of TGF-β3 were applied, suggesting that complimentary assay methodologies must be developed.

1. Introduction

Transforming growth factors β (TGF-β) are a family of structurally related dimeric proteins which exhibit a wide variety of biological actions, and in particular regulate the proliferation of epithelial cells [1]. They are unique in that each monomer contains nine strictly conserved cysteines, one of which stabilizes the dimer via an interchain disulfide bond [2,3]. The monomers, each with a molecular mass of approximately 12 500, are stabilized by two identical hydrophobic surfaces [3].

The objective of the present study was to develop an analytical reversed-phase high-performance liquid chromatography (RP-HPLC) assay for TGF-β3 which can be used to investigate the solution stability of the protein. The literature suggests that a number of variables can affect chromatographic performance for RP-HPLC of proteins [4,5]. Unlike small molecules, proteins have the added challenge that altered conformation can impart on retention. The change in conformation may occur due to interaction with the hydrophobic stationary phase, the column temperature, or the mobile phase components [5]. If more than one conformation exists during the separation, broader bands are expected relative to conditions where only on conformer exists. Thus, one strategy to improv-

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0021-9673/95/$09.50
SSDI 0021-9673(95)00452-1

ing chromatographic performance is to purposefully induce denaturation of the protein [5].

Most RP-HPLC assays for proteins utilize an acetonitrile (ACN) gradient with trifluoracetic acid (TFA) to reduce the pH and silanol ionization [4]. In addition to modifying the pH, the TFA may also act as a hydrophobic ion-pairing agent and as a denaturant [4,5]. The choice of the initial organic concentration can impact on total assay time since at low organic concentrations proteins generally remain adsorbed on the stationary phase until the appropriate concentration of organic is reached [6,7]. This results in an extremely steep negative slope when the logarithm of capacity factor is plotted versus the percent organic [8]. It has been shown that increasing the gradient rate can dramatically decrease peak width, and may lead to improved recovery [4,5]. Increasing flow-rate can improve resolution for some peptides; however, this may also lead to a significant loss in sensitivity [4]. Thus, no attempt was made to vary flow-rate as part of the present study. Effects of column temperature on protein chromatography have been proposed due to increased solubility in the mobile phase, more rapid transfer between the stationary and mobile phases, and on-column conformational changes [5,9–11]. Improvements in band width with increasing temperatures have been noted for some proteins and peptides [5,9,12]; however, this is apparently not generally true [13,14]. Temperature can also be utilized to vary selectivity [13].

Based on the above literature observations, our approach for TGF-β3 was to evaluate the effect of altering (1) the initial percent of ACN, (2) the gradient slope, (3) the percentage of TFA, and (4) column temperature in order to optimize overall chromatographic performance, and peak width in particular.

2. Experimental

Samples (at 5°C) containing 120 μg/ml of TGF-β3 were injected using a BIO-RAD Model AS-100 HRLC automatic sampling system with a 20-μl loop. The gradient for the assay was provided by the ConstaMetric 4100 solvent delivery system by LDC Analytical operated at 1 ml/min. A Vydac C_4 column (250 × 4.6 mm I.D.) and guard column were kept at a constant 45°C (except during temperature studies) using a BIO-RAD column heater. Samples were detected at 210 nm using the BIO-Dimension UV–Vis monitor. Integration was performed using the BIO-RAD HRLC system interface and the BIO-RAD Series 800 HRLC system. The acetonitrile (ACN, Fisher Scientific) and the trifluoracetic acid (TFA, J.T. Baker) were both HPLC grade. Milli-Q water was utilized for all studies. TGF-β3 (lot PO1 1409) was used as provided by Ciba Geigy.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed under non-reducing conditions using a Pharmacia Phast System (Pharmacia technique number 110). Pharmacia 8–25 gradient gels were utilized, and staining was performed with Coomassie Blue (Pharmacia technique number 200).

3. Results and discussion

An RP-HPLC assay using a C_{18} stationary phase, 0.1% TFA, and an acetonitrile gradient has previously been reported by Ogawa et al. [15] for TGF-β2.3, a heterodimer of the TGF-β2 and TGF-β3 monomers. These authors observed a peak width at half-height of approximately 1 min for this protein, which eluted at approximately 17 min with a linear acetonitrile gradient and 0.1% TFA. Preliminary attempts to assay TGF-β3 under similar conditions in our lab led to peaks with this breadth or even wider. Thus, we attempted to analyze those variables which could lead to improvements in the peak width, and thereby improve resolution from potential degradation products.

The effects of altering the initial percent organic modifier are shown in Table 1. As expected, shorter retention times were found with increased initial ACN concentrations. However, there was little or no change in peak width at half-height. Increasing TFA concentrations

Table 1
Observed changes in retention

Initial ACN conc. (%)	TFA (%)	ACN/min (%)	Temp. (°C)	Retention time (min)	Width at $\frac{1}{2}h$ (min)
35	0.1	1.0	30	10.87	1.41
32	0.1	1.0	30	12.95	1.29
30	0.1	1.0	30	15.72	1.14
27	0.1	1.0	30	18.15	1.19
25	0.1	1.0	30	20.02	1.22
27	**0.2**	1.0	30	17.82	1.19
27	**0.4**	1.0	30	20.20	0.99
27	0.1	**2.0**	30	11.56	0.60
27	0.1	**1.5**	30	12.91	0.88
27	0.1	**1.0**	30	15.37	1.26
27	0.1	**0.5**	30	22.13	3.32
27	0.1	1.0	**30**	15.81	1.17
27	0.1	1.0	**35**	16.08	0.93
27	0.1	1.0	**40**	16.12	0.70
27	0.1	1.0	**45**	15.83	0.47
27	0.1	1.0	**50**	15.93	0.23
27	0.1	1.0	**55**	15.84	0.23
27	0.1	1.0	**60**	15.75	0.23
27	0.1	1.0	**65**	15.94	0.23

Values in bold face indicate the changed values.

altered peak width to a small degree only, and complicated detection due to increased background. By increasing the gradient slope, it was found that peak width could be reduced; however, the peaks appeared to be somewhat asymmetric. Significant improvements were made in the peak shape by increasing temperature (Fig. 1). With a 20 min gradient time and a 27–47% acetonitrile linear gradient, the peak width decreased from 1.17 to 0.23 min on going from 30 to 50°C, respectively. No additional improvement was seen at 55 to 65°C.

The retention of proteins in reversed-phase chromatography is complicated by conformational changes that can occur on the column [16,17]. Hearn and co-workers [9] suggest that at a constant gradient time, significant changes in peak width are typically observed over a discrete temperature range. They attribute the temperature effects to alterations in the conformation of the protein in both the mobile and stationary phases. Our observation of a five-fold decrease in peak width over a 20°C range (30–50°C) is consistent with this hypothesis. No improvement in peak width was observed above 50°C, suggesting that conformational changes were no longer being induced.

One concern with the elevated temperatures is that degradation might occur on the column. In fact, at 50°C small shoulders were observed after the main peak which were not observed at lower temperatures (see Fig. 2). Were these due to improved resolution or to degradation? The areas of the peaks were analyzed to determine if their size increased with increasing temperature. The peak areas of the two late-eluting peaks do increase with temperature, and thus, may represent degradation of TGF-β3 on the column (Fig. 3). These data suggest that on-column degradation should be minimal if a temperature of 45°C or less is utilized. To study this further, a short-term stability study of 0.1 mg/ml drug in mobile phase under approximate elution conditions was conducted. Elution was calculated to

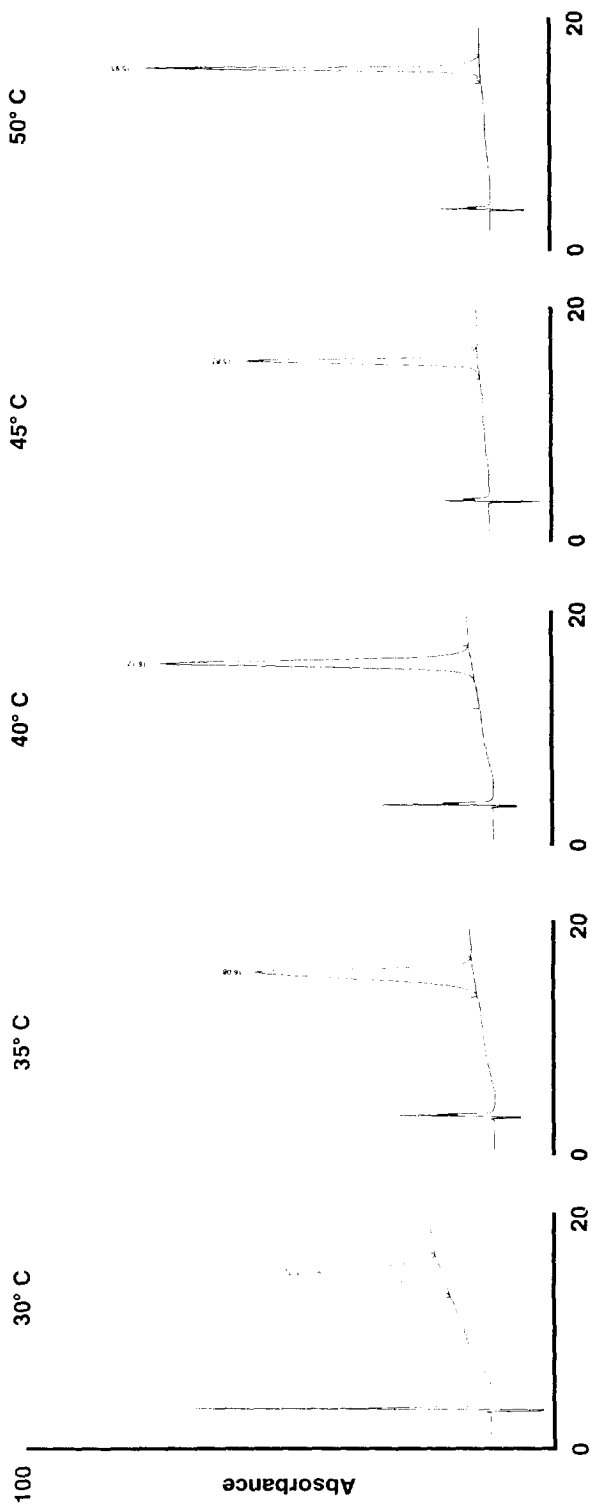

Fig. 1. Chromatograms of TGF-β3 as a function of column temperature. A gradient slope of 1% acetonitrile per min was utilized (27% initial). The detector range was two-fold higher for the 45 and 50°C conditions than for the lower-temperature conditions.

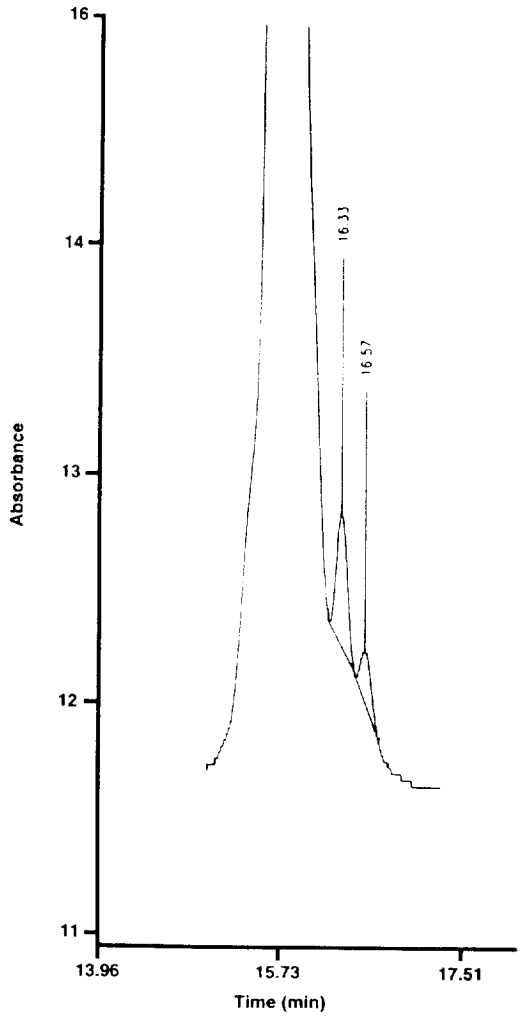

Fig. 2. Chromatogram produced at 65°C column temperature showing late-eluting shoulders which are present on TGF-β3 peak.

occur at approximately 41% acetonitrile. Thus, TGF-β3 was stored in a solution of ACN–H_2O (41:59, v/v) with 0.1% TFA at 45°C for 90 min. No increase in the area of the late-eluting shoulders was observed for up to 90 min at 45°C, which is consistent with the lack of degradation of the drug on the column. Based on the results of both studies, it appears that a 45°C column temperature achieves a balance of on-column stability and chromatographic performance. It is interesting to note that the two shoulder peaks are relatively sharp bands, suggesting that degradation occurred while the TGF-β3 was adsorbed to the stationary phase. Had the degradation occurred in the mobile phase while elution was occurring, the bands would presumably have been broader. Thus, it is possible that degradation could be minimized further by starting the separation at a higher percentage of acetonitrile.

Based on the above analysis, subsequent studies were performed using a RP-HPLC assay which utilized an initial acetonitrile level of 27%, a gradient of 1%/min for 33 min, 0.1% TFA, and a column temperature of 45°C. The other chromatographic conditions are given in the experimental section. A reproducibility study was conducted using 100 μg/ml standards prepared in 20 m*M* acetic acid buffer, 20 m*M* acetic acid in 25% acetonitrile, and 20 m*M* acetic acid in 25% isopropanol. Each sample was injected six times and the peak areas were recorded. The relative standard deviations were 7.57%, 0.75%, and 2.09%, respectively. A trend was observed in the peak areas for the aqueous standards, with peak areas increasing from the first injection to the last by 18.6%. This trend is significant and is characteristic of protein adsorption to the system. The addition of an organic solvent would appear to hinder adsorption. The linearity of the assay was investigated over the 10–150 μg/ml drug range for samples diluted with either 20 m*M* acetic acid in 25% acetonitrile or 20 m*M* acetic acid in 25% isopropanol. The standard curves for these solutions are given in Fig. 4. The correlation coefficients were 0.999 and 0.998, respectively. As a final assessment, the assay was utilized to analyze a 100 μg/ml solution of TGF-β3 in 25% isopropanol and 20 m*M* acetic acid which had been stressed under a number of conditions. These conditions included pH 12.0 (NaOH) and 25°C for 28 h, treatment with a 1000:1 molar ratio of hydrogen peroxide/protein for 6 h at 5°C, heat treatment at 95°C for 2 h, pH 2.0 (HCl) and 25°C for 28 h, and fluorescence light (intensity 6200 lm, 35°C) for 28 h. The RP-HPLC results indicate no significant drug loss for the low-pH and the light-box samples (<4% loss of potency; chromatogram not shown). However, the high-pH, peroxide, and heat-treated samples did show significant degra-

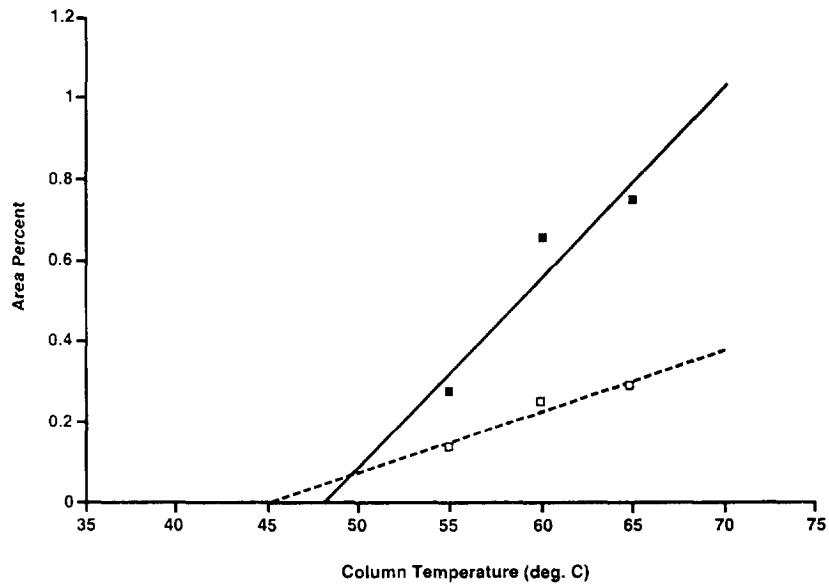

Fig. 3. Area percent of late-eluting shoulders shown in Fig. 2 as a function of column temperature. (■) Peak at 16.33 min; (□) peak at 16.57 min.

dation peaks. The chromatogram of the high-pH sample (Fig. 5) shows two broad peaks, the parent peak (conc. = 0.026 mg/ml) with a retention time of 15.12 min, and a late eluting peak with a relative retention time of 1.19 (the peak at 15.12 min was determined to be TGF-β3 by spiking the sample with additional drug). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of this sample indicated that the late-eluting peak had a molecular

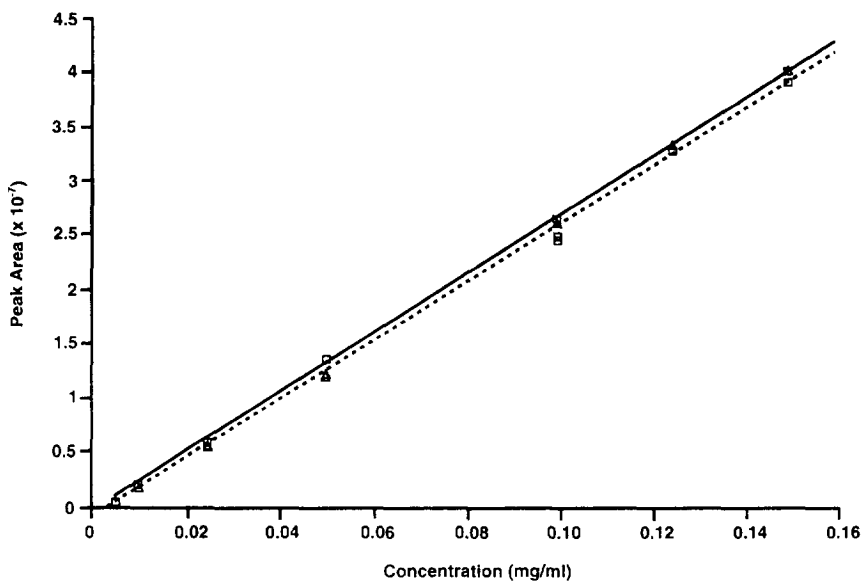

Fig. 4. Standard curves for the assay using two different sample matrices. (△) 25% Acetonitrile; (□) 25% isopropanol.

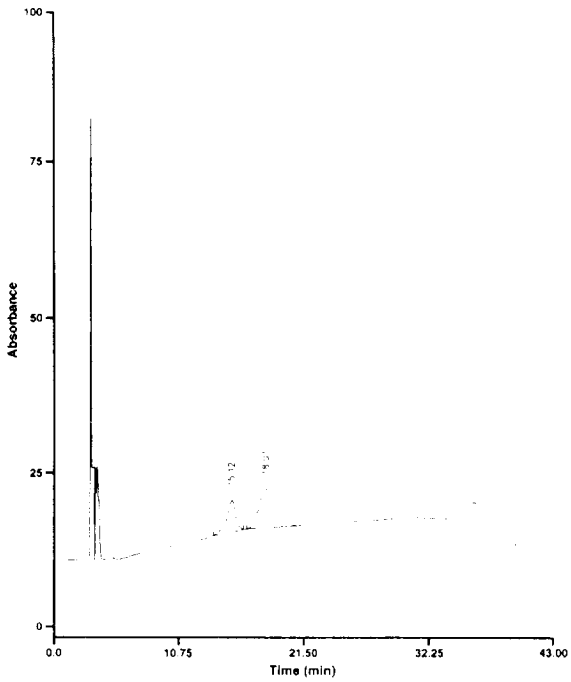

Fig. 5. Chromatogram of a TGF-β3 sample degraded at pH 12 (NaOH) and 25°C for 28 h.

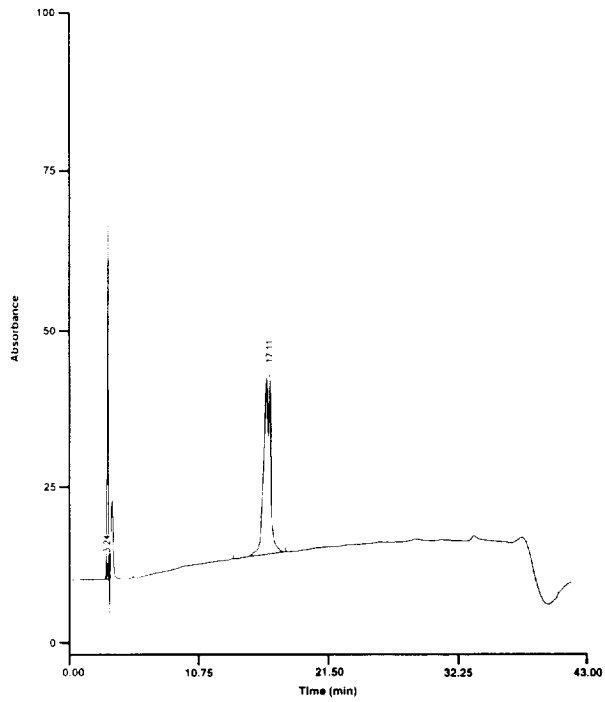

Fig. 6. Chromatogram of a TGF-β3 sample treated with excess hydrogen peroxide for 6 h at 5°C.

mass indicative of the monomer. The degradation product observed in the chromatogram for the peroxide-stressed sample (Fig. 6) has poor resolution from the TGF-β3 peak*. SDS-PAGE of this sample indicates no change in the molecular mass from that of TGF-β3, suggesting that the dimer is still present with some modification (presumably oxidation). Consistent with the temperature study performed during method development, a late-eluting shoulder was observed for the heat-treated sample (Fig. 7)[1]. The area percent of the late-eluting peak was 0.952%. As in the above stressed samples, the assay was unable to resolve this peak from the parent peak. In general, the assay does not provide baseline resolution of TGF-β3 from all potential degradation products. Thus, complimentary analytical techniques will need to be developed in order to assess all of the potential degradation pathways, as is done for most other therapeutic proteins [18].

A significant finding of this study was the fact that of all the variables studied, temperature was the most effective for yielding narrow peaks widths for TGF-β3. Although this large effect on band width might not be expected for all proteins, temperature should certainly be investigated as a method of optimizing chromatographic performance.

[1] At the time this chromatogram was obtained, the retention time for TGF-β3 had changed to just under 17 min. We believe this change occurred due to a partially clogged in-line filter, which led to a slightly reduced flow-rate (replacement of the filter returned retention times to under 16 min).

Acknowledgements

The authors gratefully thank Cheryl Kirkman and Lisa Shaner (both at Pfizer Analytical Research and Development), Lloyd Snyder (LC Resources, Orinda, CA, USA), and Milton Hearn (Monash University, Victoria, Australia) for their helpful discussions.

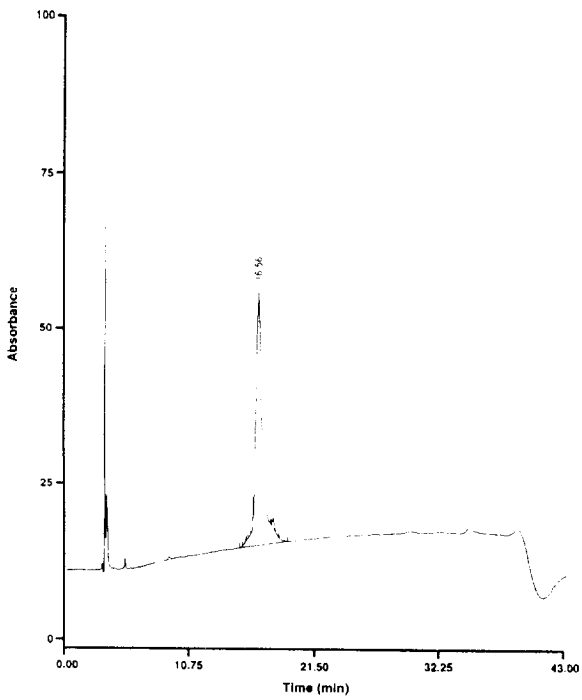

Fig. 7. Chromatogram of a TGF-β3 sample heated at 95°C for 2 h.

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