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Charge-reversed, polymer-coated capillary column for the analysis of a recombinant chimeric glycoprotein

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ABSTRACT

Fused-silica capillary columns coated with an amphiphatic polymer were used to characterize a recombinant, basic, chimeric glycoprotein. Composition, ionic strength, and pH of the electrophoretic buffers were found to affect significantly the selectivity, resolution, and rate of migration of the basic glycoprotein peaks. Out of seven buffers evaluated, sodium citrate–acetic acid buffer gave the best peak resolution while maintaining peak migration at less than 20 min. The peak resolution (R_s) was greater than 1.0 above a pH of 5.0; however, the R_s was near zero below a pH of 4.5. Peak migration time increased exponentially with increase in pH of the running buffer. The R_s increases linearly with increase in the concentration of the running buffer from 20 to 60 m*M*; the number of theoretical plates peaked at about 50 m*M* buffer concentrations. The condition selected for electrophoretic analysis of the glycoprotein uses a 50 m*M* concentration of sodium citrate-acetic acid buffer at pH of about 5.2. The standard curve for the analysis of the glycoproteins is linear over the range from 50 to 200 μg/ml glycoprotein with the number of theoretical plates over 60 000 per meter. The relative standard deviation of the assay method is approximately 4.6% and that of the peak migration time is about 3%. The polymer-coated capillary column electrophoretic analysis method has been demonstrated to be capable of monitoring degradation of the chimeric, basic FG glycoprotein.

INTRODUCTION

Analysis of recombinant proteins, especially of basic glycoproteins, by fused-silica capillary column electrophoresis presents a unique challenge due to non-specific adsorption of the proteins on the capillary wall. Unless the active binding sites on the surface of the capillary wall are masked, the proteins strongly interact with the wall resulting in severely skewed peaks or no migration of the protein. Conventionally, electrophoretic separation of proteins by the capillary column uses buffers of very low (<2.5) or very high pH (>10) to minimize the protein-to-wall interaction. However, this strategy limits the selection of an electrophoretic buffer and severely compromises peak resolution and selectivity.

Recent advancements in capillary wall modification technologies to decrease the protein–wall interactions have resulted in the commercialization of columns and column coating reagents. These technologies include coating of the capillary wall by amphipathic polymers [1], inclusion of zwitterions in the running buffer [2], and partially deactivating columns by derivatization with hydrocarbons and neutral hydrophilic compounds [3].

Samples of a recombinant glycoprotein used in this experiment were manufactured by The Upjohn Company (Kalamazoo, MI, USA). The glycoprotein, FG, is a chimeric protein composed of the fusion protein (F) and the receptor protein (G) of respiratory syncytial virus (RSV) [4]. The terms F and G are the designation of peptide segments in the RSV glycoprotein. The portion of the genes coding for the anchor region of the F and G gene were truncated and the remaining portions of the genes fused. The FG glycoprotein contains amino acids 1 to 489 of F and 97 to 279 of G and the amino acid sequence translated from the DNA sequence is presented in Table I. The F portion of FG consists of

TABLE I

PRIMARY AMINO ACID SEQUENCE OF THE CHIMERIC FG GLYCOPROTEIN OF THE RESPIRATORY SYNCYTIAL VIRUS

1	MELLILKANA ITTILTAVTF CFASGQNITE EFYQSTCTAV SKGYLSALRT
51	GWYISVITIE LSNIKENKCN GTDAKVKLIK QELDKYKNAV TELQLLMQST
101	PATNNRARRE LPRFMNYTLN NAKKTNVTLS KKRKRRFLGF LLGVGSAIAS
151	GVAVSKVLHL EGEVNKIKSA LLSTNKAVVS LSNGVSVLTS KELDLKNYID
201	KQLLPIVNKQ SCSISNIETV IEFQQKNTRL LEITREFSVI AGVTTPVSTY
251	MLTNSELLSL INDMPITNDQ KKLMSNNVQI VRQQSYSIMS IIKEEVLAYV
301	VQLPLYGVID TPCWKLHTSP LCTTNTKEGS NICLTRTDRG WYCDNAGSVS
351	FFPQAETCKV QSNRVFCDTM NSLTLPSEVN LCNVDIFNPK YDCKIMTSKT
401	DVSSSVITSL GAIVSCYGKT KCTASNKNRG IIKTFSNGCD YVSNKGVDTV
451	SVGNTLYYVN KQEGKSLYVK GEPIINFYDP LVFPSDEFDQ LGISPSNPSE
501	ITSQITTILA STTPGVKSTL QSTTVKTKNT TTTQTQPSKP TTKQRQNKPP
551	SKPNNDFHFE VFTFVPCSIC SNNPTCWAIC KRIPNKKPGK KTTTKPTKKP
601	TLKTTKKDPK PQTTKSKEVP TTKPTEEPTI NTTKTNIITT LLTSNTTGNP
651	ELTSQMETFH STSSEGNPSP SQVNISSQRE D

a F1 (48 000 dalton) and a F2 (20 000 dalton) subunit which are connected by a disulphide bridge. The FG is heavily glycosylated and appears on a sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) slab-gel to migrate at about 100 000 daltons. The FG glycoprotein is produced by infecting an insect cell, SF-9 (DT), with a recombinant baculovirus vector containing the FG gene [4]. The FG protein is excreted into the cell supernate. The G glycoprotein is associated with human immunoresponse [5]. The p*I* of the FG glycoprotein is *ca.* 9.4.

The chimeric FG glycoprotein is being developed at Upjohn as a vaccine for RSV. RSV is the primary etiological agent for lower respiratory disease in children under two years of age. Most children by the age of five will have experienced three episodes of RSV related disease. The disease occurs worldwide and mortality rates can be higher in those countries with poor health care. RSV also has been associated with respiratory disease in the elderly especially those residing in nursing homes.

The purpose of this paper is to develop a stability indicating method for the assay of a basic glycoprotein in its native form by use of surface modified capillary columns. Surface-modified columns have potential of greater peak selectivity and resolution of basic proteins unattainable by the conventional bare fused-silica capillary column.

EXPERIMENTAL

HPCE instrumentation

A P/ACE System 2100 high-performance capillary electrophoretic (HPCE) instrument (Beckman, Palo Alto, CA, USA) was used throughout the study. Each HPCE run involves a 5-s nitrogen pressure (0.5 p.s.i.) injection of a sample into a coated capillary column. The compounds migrating in the column were monitored at 214 nm. The temperature in a column cartridge was maintained at 25°C with a circulating coolant and an electrophoretic run was conducted at a constant voltage of −20 kV (−350 V/cm). The area under the protein peak was integrated by means of a program residing in a VAX mainframe computer.

Reagents

Buffers used for the electrophoretic analysis were made of analytical reagent-grade chemicals obtained from Baker (Phillipsburg, NJ, USA), Mallinckrodt (Paris, KY, USA), and Aldrich (Milwaukee, WI, USA). Buffer solution was prepared by mixing two 100 m*M* solutions, *e.g.*, sodium citrate and acetic acid, to a desired pH. The amphipathic polymer column coating reagent (Micro-Coat protein analysis reagent) [1] was obtained from Applied Biosystems (ABI, Foster City, CA, USA). Following the direction of ABI, the polymeric column coating reagent was reconstituted in an aqueous solution containing 2% ethylene glycol.

Preparation of a polymer-coated column

A roll of fused-silica capillary tubing (75 μm I.D. × 375 μm O.D.) was obtained from Polymicro Technologies (Phoenix, AZ, USA). Capillaries were cut to few centimeters longer than the final column length of 57 cm and a window was created at just over 7 cm from one end by removing the polyimid coating with a polyimid stripper (Model S200, Polymicro). The tubing was mounted in the column cartridge (Beckman) and cut to an effective column length of 50 cm. The capillary column was treated with a 0.1 *M* NaOH for 5 min. After rinsing with water for 3 min, the column was coated with the polymeric reagent by filling the capillary for 1 min

under high nitrogen pressure (20 p.s.i.) followed by 5 min under low pressure (0.5 p.s.i.). The polymeric reagent was allowed to contact statically with the column for 15 min prior to sample analysis. Just before each sample analysis, the column was briefly coated with the polymeric reagent for 0.5 min and rinsed for 2 min with the running buffer.

Capillary columns bonded with C_{18} and C_8 were purchased from Supelco (Bellefonte, PA, USA). A window was created by treating the capillary with hot nitric acid and the column was mounted in a column cartridge (Beckman) prior to an electrophoretic run.

Separation of glycoproteins

The chimeric FG glycoprotein used in this study (Upjohn) was analyzed at a concentration of about 80 μg/ml in the native form by dilution in water or as denatured by dilution in 0.1% trifluoroacetic acid (TFA). After a brief coating with the polymeric agent (0.5 min), the column was rinsed for 2 min with a 50 m*M* sodium citrate–acetic acid buffer (pH 5.2). The sample was injected into the column by nitrogen pressure (0.5 p.s.i.) for 5 s and analyzed for 15 min under a constant voltage of −20 kV (130 μA).

RESULTS AND DISCUSSION

Polymeric, amphipathic column coating reagent

The polymeric, amphipathic column coating reagent used is a neutral lipophilic polymer molecule containing a hydrophilic group on a side chain [1]. The polymer coats the wall of the capillary column through an ionic interaction with the silanol group. The polymeric molecule immobilizes the hydrophilic group forming a stable, net positively charged amine layer on the capillary wall surface. The positively charged column reverses the electroosmotic flow to the anode.

Positive charge on the capillary surface minimizes non-specific binding of positively charged proteins to the wall. Thus, the amphipathic polymer-coated, positively charged column allows manipulation of net charges of proteins by selection of a wide variety of buffers to fine-tune electrophoretic conditions for the separation of proteins. Unlike when using zwitterions [2], the buffer contains no column coating reagent. In our experience, the amphipathic polymer-coated column is stable for one electrophoretic run and the column must be briefly recoated with the reagent just before each analysis in order to attain a reasonable precision of peak migration time.

Selection of buffer

Variety of buffers, all at 20 m*M* concentration and pH of approximately 5.2, were prepared: sodium acetate–acetic acid, sodium citrate–acetic acid, sodium citrate–citric acid, sodium acetate–phosphoric acid, sodium citrate–phosphoric acid, sodium phosphate–phosphoric acid, and sodium citrate–formic acid. These buffers were used to examine peak resolution and selectivity for the analysis of the chimeric FG glycoproteins.

Migration of a single FG glycoprotein peak was noted when sodium acetate–acetic acid, sodium citrate–citric acid, sodium acetate–phosphoric acid, and sodium citrate–phosphoric acid were used as the running buffers. No FG glycoprotein peak was detected when a sodium phosphate–phosphoric acid buffer was used. Presence of 2 peaks was observed in a crude preparation of the FG glycoprotein when sodium citrate–acetic acid and sodium citrate–formic acid buffers were used. The sodium citrate–acetic acid buffer was chosen for further study since the peak migration time was less than 20 min. The peak migration time of the FG glycoprotein was twice as long with inferior peak resolution when the sodium citrate–formic acid buffer was used.

When the phosphate buffer at pH 2.5 was used with an untreated column, no migration of the FG glycoprotein was noted. A single FG peak was detected by use of a borate buffer at pH 11.

Effect of pH on peak resolution

To study the effect of pH on peak resolution (R_s), a 100 m*M* concentration of sodium citrate and acetic acid was prepared and mixed to obtain pH values of 4.0, 4.5, 5.0, 5.5, 6.0 and 7.0. These buffer solutions were diluted to 50 m*M* just before use.

As shown in Fig. 1, the R_s improved dramatically from near zero to over 1.0 between the pH of 4.5 and 5.0. Only a gradual increase in R_s was noted above the pH of 5.0. The R_s remained near zero below the pH of 4.5. Increase of the R_s follows the equation $y = 0.0199x^{4.26}$, where y represents the R_s and x represents the pH values.

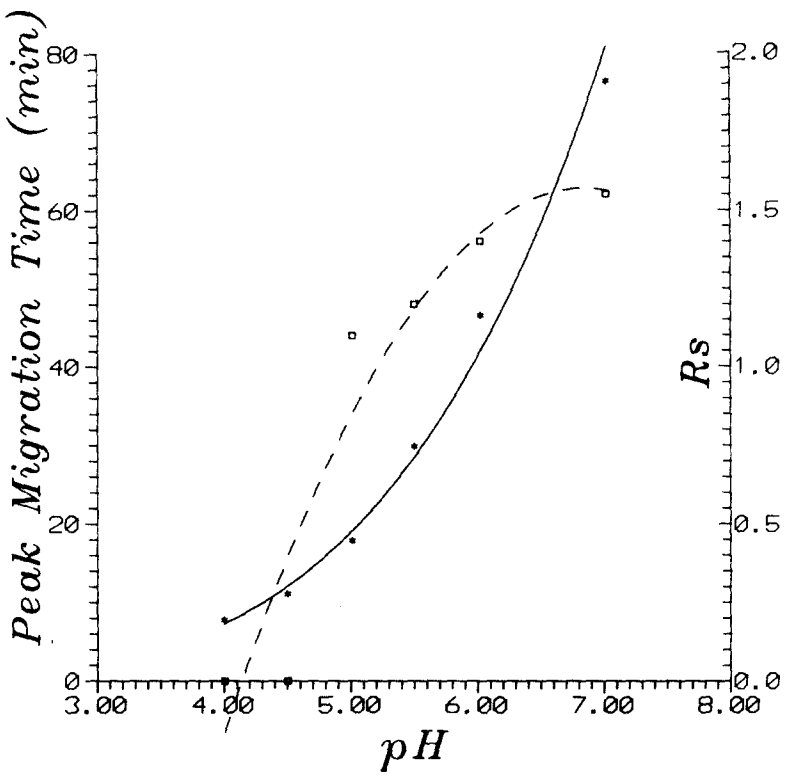

Fig. 1. Effects of pH of the running buffer on resolution (R_s) (□) and migration (∗) of the chimeric FG glycoprotein peaks. Conditions: −350 V/cm; column temperature, 25°C; migration distance, 50 cm; polymer-coated fused-silica capillary column, 75 μm I.D.; running buffer, 20 m*M* sodium citrate–acetic acid.

Unlike in conventional capillary electrophoresis, the direction of eletroosmotic flow in a polymer-coated, positively charged column is toward the anode. The rate of the reversed electroosmotic flow increases by lowering the pH of the buffer. Since the electroosmotic flow is inversely proportional to the pH of the running buffer, it was not surprising that the peak migration time increased exponentially with increase in pH. A running buffer with a pH of between 5.0 and 5.5 was selected for further study in order to consistently attain an R_s value of over 1.0 while maintaining the peak migration time at less than 20 min.

Effect of buffer concentration on peak resolution and theoretical plates

A 100 m*M* concentration of sodium citrate–acetic acid buffer at pH 5.2 was diluted to 20, 30, 40, 50, 60, 70 and 80 m*M* to examine effects of the buffer concentration on the R_s and the number of theoretical plates (N). The results are presented in Fig. 2.

The R_s increased linearly with an increase of the buffer concentration from 20 to 60 m*M*. The increase followed the linear equation $y = 0.031x - 0.41$, where y represents the R_s value and x represents the buffer concentration in m*M*. The R_s, however, suddenly dropped to near zero when the buffer concentration increased beyond the concentration of 70 m*M*. Increase of the buffer concentration proportionally increased the flow of the electric current. The increase of the electric current followed the linear equation $y = 1.26x + 0.174$ ($r > 0.999$), where y represents current in mA and x represents the buffer concentration in m*M*.

Increase in the apparent N value peaked at about 60 000/m with a buffer concentration of approximately 40 to 50 m*M*. This phenomenon, a sharp decrease of N above a buffer concentration of

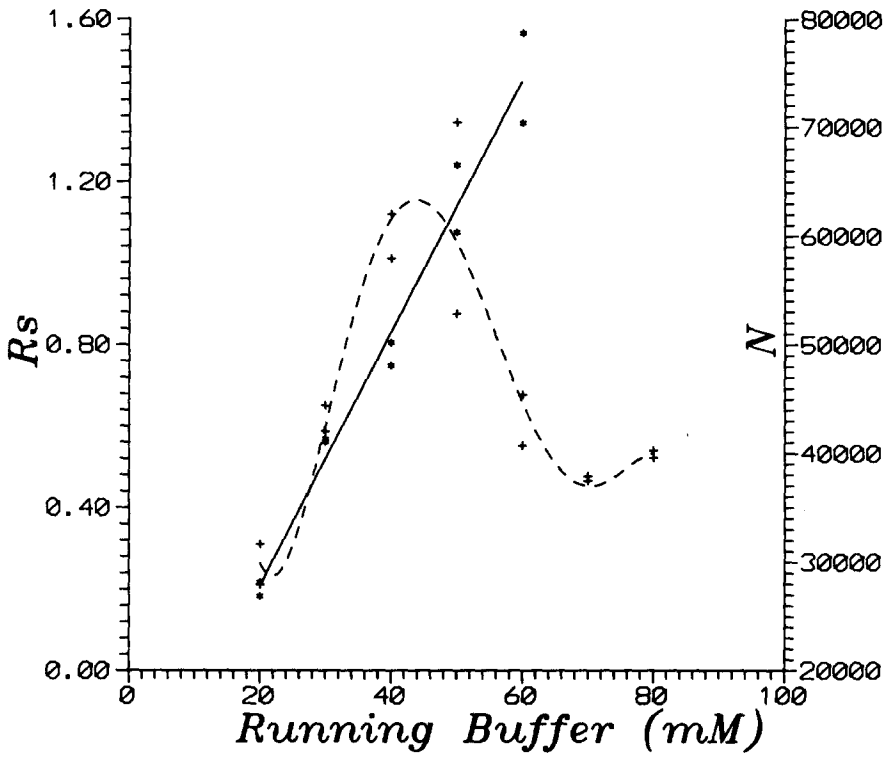

Fig. 2. Effects of concentration of the running buffer on resolution (R_s) (*) and number of theoretical plates (N) (+) of the chimeric FG glycoprotein peaks. Running buffer, pH 5.2 sodium citrate–acetic acid; conditions as in Fig. 1.

50 mM, was not observed when another basic protein, sCD4, was tested. Thus, in addition to the high salt concentration and the Joule heating, the microheterogeneities of carbohydrate moieties in the FG glycoprotein may also be contributing to the phenomenon. Since the FG glycoprotein is indicated to be extensively glycosylated and contains both N- and O-glycosylation sites (data not shown), increase in N of a mere 10 000 to 20 000/m would not be sufficient to separate each and every FG glycoprotein form. Although the observed N value decreased, it may be theorized that it actually increased above the buffer concentration of 50 mM. The polymer coated column, in reality, tried to separate the glycoproteins containing minor differences in the carbohydrates. The net effects, therefore, were broadening of the FG glycoprotein peaks. Indeed, the FG protein appeared as a diffused peak by the SDS-polyacrylamide gel-filled capillary electrophoresis column [6] and as a smeared band on a SDS-PAGE slab gel. Thus, increase in the true N value caused an apparent broadening of the FG peak which in turn resulted in a sharp drop in the observed N value and loss of the R_s value. Tran *et al.* [7] successfully split a glycoprotein, erythropoietin, into four peaks by use of a very short column with an exact capillary zone electrophoresis condition.

Since the buffer concentration showed minimum effect on the electroosmotic flow of the polymer-coated capillary column, effect of the buffer concentration on peak migration time was minimal.

The following conditions for the polymer-coated capillary column electrophoretic separation were considered optimum for the analysis of the chimeric FG glycoproteins: running buffer, 50 mM sodium citrate–acetic acid at a pH of *ca.* 5.2; polymer-coated fused-silica capillary column, 75 μm I.D.; effective column length, 50 cm; column temperature, 25°C; constant voltage at −350 V/cm.

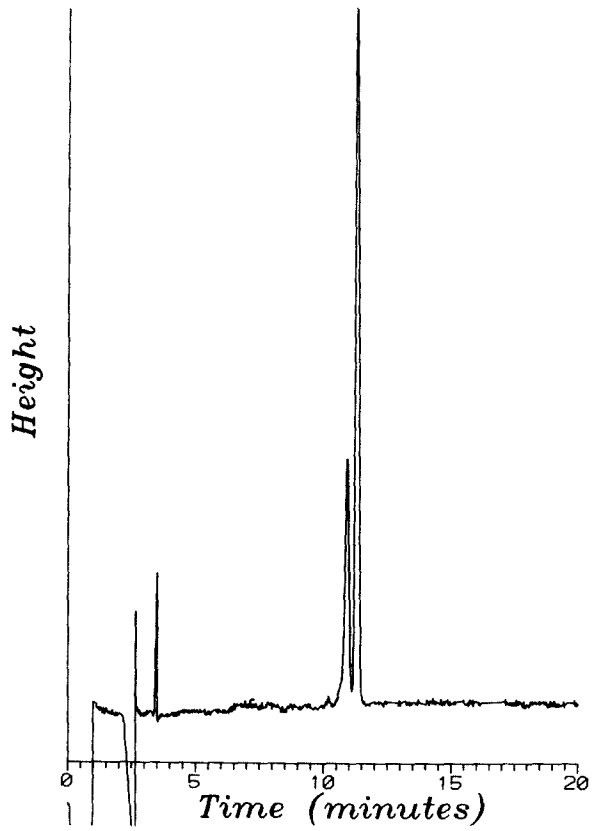

Fig. 3. Capillary electrophoretic separation of the chimeric FG glycoproteins of the respiratory syncytial virus using a polymer-coated fused-silica capillary column as monitored at 214 nm. Running buffer, 50 m*M* sodium citrate–acetic acid at pH 5.2; conditions as in Fig. 1.

Capillary electrophoretic analysis of the chimeric FG glycoproteins

A typical electropherogram indicating separation of peaks in the chimeric FG glycoprotein is presented in Fig. 3. The standard curve for the analysis of the FG glycoprotein is linear ($r > 0.996$) between the glycoprotein concentration of 50 and 200 μg/ml (Fig. 4). A linear regression equation follows $y = 15\,300x - 546\,000$, where y represents the peak area and x represents the glycoprotein concentration in μg/ml. The curve intersects the x axis at about 35 μg/ml glycoprotein indicating adsorption of the glycoprotein on the capillary wall. The adsorption of the glycoprotein may be due to incomplete coating of the capillary wall with the polymeric agent.

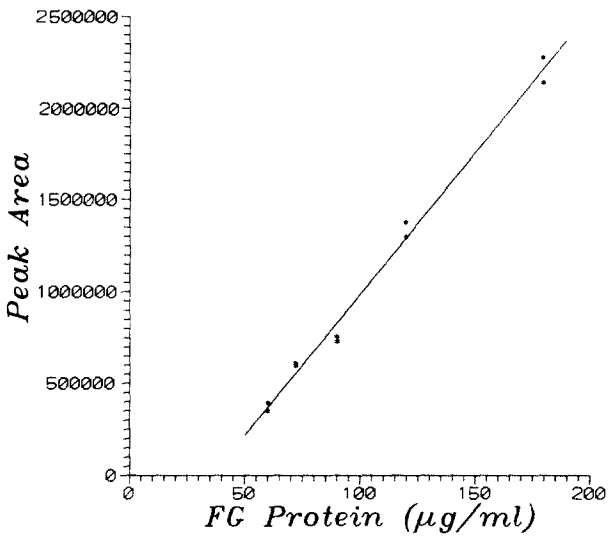

Fig. 4. Standard curve for the electrophoretic analysis of the chimeric FG glycoprotein using a polymer-coated fused-silica capillary column. Peaks migrated were monitored at 214 nm. The curve indicates existence of a linear relationship ($r > 0.996$) between the weight of the FG glycoprotein and the peak area. Running buffer, 50 m*M* sodium citrate–acetic acid at pH 5.2; conditions as in Fig. 1.

The precision for the assay of the biotechnology-derived chimeric FG glycoprotein of RSV was evaluated by repeated injection of the glycoprotein sample at *ca.* 80 μg/ml concentration. The relative standard deviation (R.S.D.) for the assay of the glycoprotein was about 4.6% and that of the peak migration time was approximately 3.0% (Table II). The R.S.D. of the peak migration time obtained in this study was approximately twice that of a capillary zone electrophoretic assay method [8]. This high R.S.D. may be caused by difficulties in obtaining a consistent/homogeneous coating of the column allowing a minor, inconsistent binding of the glycoprotein on the column surface.

A crude FG glycoprotein preparation contaminated with a trace amount of a proteolytic enzyme was diluted with water and incubated in the native form at about 30°C. The sample was assayed at 60-min intervals. Fig. 5 depicts the electropherograms when the sample was analyzed after 0, 3 and 6 h of incubation. Appearance of a peak with corresponding decrease of the peak, numbered as 2, was noted. The peak, numbered as 1, was unaffected by the treatment. Thus, the polymer-coated capillary

column electrophoretic method may be of value to assess stability characteristics of the chimeric FG glycoprotein. Identification of the peaks which appeared in the electropherogram awaits the successful interfacing of a capillary electrophoretic instrument with a mass spectrometer.

An FG glycoprotein sample was denatured by diluting the sample with a 0.1% TFA solution. A 40-μl quantity of the denatured sample was pipetted onto a polyolefin microvial (Beckman). The microvial was then placed with a vial spring in a glass vial (4 ml capacity) containing 2 ml water and capped with a silicon vial cap (Beckman). Water was added to the vial to minimize evaporation. When the sample was analyzed at 60-min intervals, a progressive increase in the peak area was noted (Fig. 6). The peak area nearly doubled within 250 min while the sample was waiting in a sample tray for analysis. The sample vial was sealed with a silicone rubber vial cap, but the cap has a crosswise cut on its top for easy insertion of the capillary column for injection of a sample. The instrument has no provision for cooling samples and the sample vials easily reach 30°C. Thus, increase in the peak area during the prolonged assay period may be caused by evaporation of the sample solution through the cut made in the vial cap. Inclusion of an internal standard is essential to correct for sample evaporation.

TABLE II

PRECISION OF THE HIGH-PERFORMANCE CAPILLARY ELECTROPHORETIC ANALYSIS OF A CHIMERIC FG GLYCOPROTEIN USING A POLYMER-COATED FUSED-SILICA CAPILLARY COLUMN

Peaks migrating were monitored at 214 nm. Conditions: −350 V/cm (85 μA); column temperature, 25°C; migration distance, 50 cm; column 75 μm I.D.; running buffer, 50 m*M* sodium acetate–citric acid at pH 5.2.

Run No.	Peak migration (min)	FG glycoprotein peak area
1	10.24	1 226 000
2	9.74	1 168 000
3	9.78	1 259 000
4	9.79	1 343 000
5	9.86	1 311 000
6	9.87	1 247 000
7	9.29	1 247 000
8	9.33	1 351 000
9	9.39	1 285 000
Average	9.70	1 271 000
R.S.D. (%)	3.18	4.60

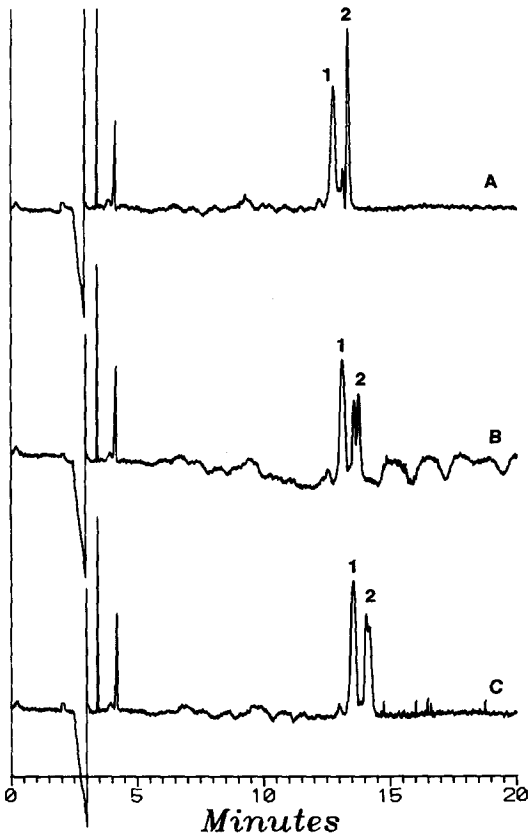

Fig. 5. Composite electropherograms of a crude chimeric FG glycoprotein sample indicating formation of a degradation compound when held at 30°C. Electropherograms: A, 0 h; B, 3 h; C, 6 h at 30°C. Sample diluted to 60 μg glycoprotein per ml water. Running buffer, 50 m*M* sodium citrate–acetic acid at pH 5.2; conditions as in Fig. 1.

Appearance of a new peak, as observed in the FG sample without TFA (Fig. 5), was not noted. This was expected as TFA solution inactivates proteolytic enzymes.

C_8 and C_{18} derivatized column

Capillary columns derivatized with C_8 and C_{18} were examined for the analysis of the chimeric FG glycoprotein. A single, highly skewed FG glycoprotein eluted from the columns. Variety of buffers with differing pH values were used in an attempt to

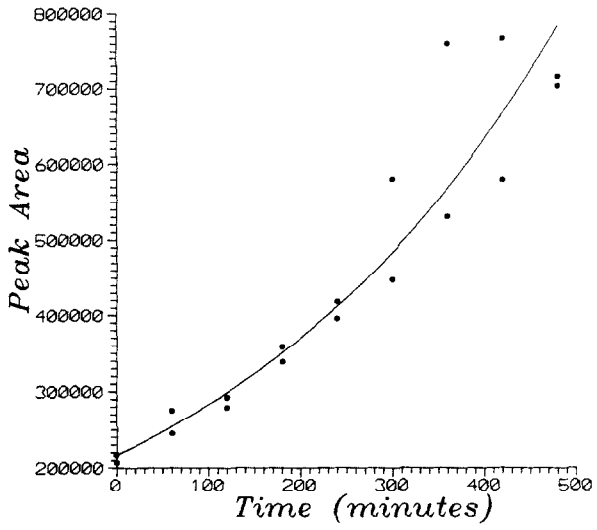

Fig. 6. Peak area of a chimeric FG glycoprotein analyzed at 60-min intervals indicating evaporation of the sample solution.

improve selectivity of the column. No improvement was attained.

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