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One-step capillary isoelectric focusing for the separation of the recombinant human immunodeficiency virus envelope glycoprotein glycoforms

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Abstract

One-step capillary isoelectric focusing was investigated as a rapid method to resolve the glycoforms of the heterogeneous recombinant human immunodeficiency virus (HIV) envelope glycoprotein (rgp 160sMN/LAI). The separation was performed in a poly(vinyl alcohol) (PVA) coated capillary using a mixture of ampholyte of narrow and wide pH range. A combination of saccaharose and 3-(cyclohexylamino)-1-propanesulfonic acid was shown to be the most efficient additive to avoid protein precipitation which occurs at a pH close to its p*I*. Although the calibration curve [isoelectric point (p*I*) vs. migration times] showed a non-linear relationship, an adequate linearity could be yielded for short p*I* ranges permitting to exhibit the acidic character of the different glycoforms of the rgp 160s MN/LAI (p*I* from 4.00 to 4.95). Reproducibility evaluated by comparing the performance of a polyacrylamide and a PVA coated capillary showed that low RSD values were obtained for intra-day (0.5 to 1.9%) and inter-day (1.6 to 7.6%) measurements using the PVA capillary. Moreover, the long term stability of the PVA capillary was demonstrated by measuring the variation of migration times of the protein markers for a long period of use. Finally, this method was able to differentiate the glycoform pattern of two close glycoproteins such as the rgp 160 of two sub-populations of the virus HIV-1.

Keywords: Isoelectric focusing; Human immunodeficiency virus; Glycoproteins; Proteins

1. Introduction

An increasing number of proteins of therapeutic value are now produced by recombinant cells. Process development, production monitoring as well as quality control require rapid and efficient techniques for protein analysis. On the one hand, phosphorylation or glycosylation of recombinant proteins depend on the cell culture conditions, on the nature and the age of the host cells, and on the purification processes [1,2]. On the other hand, these post translational modifications of proteins may affect their biological activity, immunogenicity, clearance rate, solubility, stability and protease resistance [1,3]. That is why the monitoring of both the heterogeneity of carbohydrates and the consistency of glycosylation from lot-to-lot is a fundamental requirement in the quality control of recombinant glycoproteins.

At the moment, a high priority is to better understand the mechanisms and propagation of

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human immunodeficiency virus type-1 (HIV-1). One of the strategies to develop an efficient anti HIV-1 vaccine has been to produce recombinant HIV envelope glycoprotein (rgp 160) [4–7] or its external part gp 120 [8–11] which are considered as potential vaccines. In addition to having *O*-linked structures, *N*-glycans can account for up to 50% of the mature gp 160 [12] exhibiting a molecular mass of 160. As reported in a previous study, *N*-linked carbohydrates of gp 120 were shown to be equally represented by both high mannose and complex sialic acid-containing carbohydrate glycans [13]. In the present work, the charge heterogeneity of a soluble recombinant gp 160 variant derived from the natural HIV-1 envelope protein described by Kieny et al. [14] and Taverna et al. [15] has been studied.

The traditional method to analyse charge heterogeneity of proteins and especially glycoforms on the basis of their sialic acids content has been isoelectric focusing (IEF). Capillary IEF (cIEF) offers the potential to combine the high resolving power of conventional gel IEF with the advantage of an automated capillary electrophoresis instrument.

In cIEF, the separation of charged analyte molecules takes place in a pH gradient created in a capillary by carrier ampholytes under the influence of an electric field. After the introduction of the sample–ampholyte into the capillary and once the focusing step has been completed, there is in theory no further movement in the capillary. During this step, discrete sample bands begin to form into a single zone at the protein isoelectric point (p*I*). This is accompanied by a decrease in the current generated, reaching a minimum value at the completion of the focusing step. A mobilisation step is thus required to drive the focused zones past the detector. To overcome excessive band broadening during this additional step, two cIEF techniques have been essentially developed. In the 'two-step' method, focusing and mobilisation take place sequentially, the latter is achieved independently after the focusing step, either by hydrodynamic force (pressure or gravity mobilisation) or by replacing the catholyte or the anolyte by a solution containing a salt or a zwitterionic mobilising agent [16] (chemical mobilisation). In the 'one-step' method, the components are mobilised simultaneously with the focusing, by means of the residual electroosmotic flow (EOF).

Several reviews have summarised the contribution of cIEF for (glyco)proteins analysis [17–19]. Rodriguez-Diaz et al. have recently described in detail the mechanism of cIEF [20,21]. Other studies concerning the evaluation of carrier ampholytes [22,23], the effects of catholytes on chemical mobilisation [24] have been recently published.

One of the first attempts to apply cIEF to a glycoprotein was published by Kilar and Hjerten [25,26] for the separation of human transferrin isoforms. Later, a similar application was reported, this time for a recombinant glycoprotein by Yim et al. [27] studying the fractionation of the glycoforms of recombinant tissue plasminogen activator (rt-PA).

Mazzeo and Krull [28] have introduced as early as 1991 a rapid method designed 'one-step cIEF' permitting both focusing and mobilisation to take place simultaneously, where run times were reduced from 30 min for a 'two-step cIEF' to 5 min through reversing the polarity and shortening the separation distance. The EOF was maintained in the capillary but controlled by the addition of methyl cellulose (MC), and *N*,*N*,*N′*,*N′*-tetramethylethylenediamine (TEMED) was added to block the detector-distal capillary segment. Since then, this 'one-step cIEF' approach has been exploited by Moorhouse et al. [29] to further improve the separation of the rt-PA glycoforms in less than 10 min.

Schwer [30] demonstrated that cIEF may be applied for routine analysis of protein samples in quality and purity control. In particular, single-step cIEF with EOF mobilisation and two-step cIEF with pressure or chemical mobilisation have been compared in terms of linearity, resolution and reproducibility. These three cIEF techniques showed satisfactory reproducibilities. One-step cIEF provided shortest analysis times but correlation of p*I* with migration time was linear only over a narrow range of pH as previously discussed by Mazzeo and Krull [28]. In contrast, two-step cIEF with pressure mobilisation required the use of high electric field during mobilisation to compensate for the loss of resolution. Chemical mobilisation provided a good linearity of p*I* vs. migration times and exhibited the highest resolution but longer analysis time. Since then, a growing number of publications have emphasized the potential of cIEF for the quality control of recombinant products [31–33].

The validation issues of cIEF methods for the monitoring of charge heterogeneity of recombinant glycoproteins have recently appeared. One of the first investigations to validate a method was published by Chen et al. [34] who also evaluated the ruggedness of the method by evaluating four commercially available carrier ampholytes for their ability to resolve rt-PA glycoforms. In particular, a detailed validation of a one-step cIEF for the electrophoretic separation of rt-PA was attempted by the same group in 1996 [35]. In 1998, Hunt et al. [36] have developed and validated in accordance with ICH guidelines a cIEF method for determining the identity and the charge distribution of a recombinant monoclonal antibody to human CD20 antigen (C2B8). The results of the different parameters studied have proven this method to be accurate, precise, linear, highly specific, rapid and suitably robust for testing the bulk and final product.

The most recent development of cIEF relies in a new fractionated injection protocol called the 'sandwich injection' proposed by Kilar et al. [37] allowing the separation of compounds having p*I* values outside the pH range of the ampholytes employed.

Precipitation of proteins at a pH close to their p*I* is one of the main problems encountered in cIEF. The rationale of protein solubilisation has been discussed by Rabilloud [38] in a review, examining the nature of the possible interactions involved. Various strategies have been described in the literature to overcome precipitation during focusing of proteins. Urea and ionic detergents [e.g. sodium dodecyl sulfate (SDS)] are efficient [34,39,40] but these denaturant conditions make them not always applicable. Several authors have investigated the use of a large number of solubilisers: non-detergent sulfobetaines, non-ionic detergents (Triton X-100, Nonidet P-40), zwitterionic detergents {taurine, 3-[(3-cholamidopropyldimethylammonio]-1-propane sulfonate (CHAPS)}, Good's buffer [bicine, 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS)], neutral additives (sucrose, sorbose, sorbitol, glycerol, ethylene and propylene glycol) have been successfully employed alone or in combination [41,42]. Another agent, *n*-octylglucoside, was exploited by Schmerr et al. [43] to assist the solubilisation of the scrapie prion protein during cIEF. In addition, Liu et al. [44] summarised in a detailed review the desirable properties of a carrier ampholyte and its influence on the protein solubility during the focusing step.

In the present study, we have investigated the one-step cIEF approach for the analysis of the recombinant gp (rgp) 160s-MN/LAI with the aim to separate the population of glycoforms and to compare the rgp 160 of two sub-populations of the virus originating from Europe or from Asia called clades B and E virus, respectively. Preliminary attempts to separate the glycoforms of rgp 160 using capillary zone electrophoresis (CZE) at acidic or alkaline pH values remained unfruitful (unpublished data). Only a single and broad peak was observed using a borate buffer. One of the main problems encountered with this protein is its poor solubility at pH close to its p*I* and its tendency to self aggregate. We have therefore studied the influence of several parameters such as the effect of different classes of solubilising agents, the role of the concentration and pH range of the ampholyte as well as the nature of coated capillaries and the voltage applied on the separation of rgp 160 glycoforms. An evaluation of the precision of this method was performed not only through intra- and inter-day measurements but also through a reproducibility study of a single capillary over a long period of use.

2. Experimental

2.1. Reagents and proteins

Uncoated and coated [poly(vinyl alcohol) (PVA) or polyacrylamide (PAA)] fused-silica capillaries, cIEF gel, carrier ampholyte pH 3–10 and the protein markers ribonuclease A (RNase A, p*I* 9.45), carbonic anhydrase II (CAH II, p*I* 5.9), β-lactoglobulin A (β-LGA, p*I* 5.1) and cholecystokinin flanking peptide (CCK, p*I* 2.75), were obtained from Beckman Instruments (Fullerton, CA, USA). All other chemicals, including ampholines pH 3.5–5, 5–8, 3.5–10, myoglobin (Mb, p*I* 6.8, 7.2) and trypsin inhibitor (TI, p*I* 4.6), 1 *M* phosphoric acid, CAPS, 2-(*N*-morpholino)ethanesulfonic acid (MES), taurine, tricine, urea, reduced triton X-100, Nonidet P-40, sorbitol, saccharose, ethylene glycol, poly(ethy1ene glycol) (PEG) were purchased from Sigma (St. Louis, MO, USA). 1 *M* sodium hydroxide was from

Prolabo (Fontenay-sous-bois, France). Glycerol was obtained from BDH (Poole, UK). Hydroxypropylmethylcellulose (HPMC) was from Seppic (France). TEMED was from Pharmacia (Uppsala, Sweden). Tween-80 was from Difco (Detroit, USA).

A recombinant soluble gp 160s MN/LAI variant (rgp 160) was produced by Pasteur Merieux Connaught (Marcy–l'Etoile, France). The glycoprotein was desalted by gel filtration (Bio-Gel P2 from Bio-Rad), dried by lyophilisation and stored at −20°C until use.

All solutions were prepared using deionised water from Milli-Q and were filtered through a 0.20 μm membrane (Millex, Millipore, France) prior to CE analyses.

2.2. Capillary electrophoresis

cIEF was performed using a P/ACE 5500 instrument with UV detector and equipped with a capillary cartridge of 75 μm I.D.×375 μm O.D. (Beckman). The absorbance of the focused proteins was detected at 280 min. The capillary was thermostated at 20°C. Samples were diluted to a concentration of 200 to 900 ng/μl in a mixture of ampholyte and solubiliser agents and then applied by filling a part of the capillary. Before each injection the capillary was rinsed for 1 min with water at 20 p.s.i. (1 p.s.i.= 6894.76 Pa). Between runs the capillary was flushed with water and 10 m*M* H_3PO_4 for 2 min each.

One-step cIEF method experiments were performed in the reversed polarity mode with 20 m*M* NaOH as the catholyte at the capillary inlet and 100 m*M* H_3PO_4 as the anolyte at the outlet. Uncoated PVA or PAA coated capillary (50 μm I.D.) with an effective length of 40 cm (total length 47 cm) were filled, by low pressure (0.5 p.s.i.) during 0.4 s at the capillary outlet, with 0.2 to 0.9 μg of rgp 160 per μl, 5% pH 3.5–5, pH 5–8 and pH 3–10 ampholytes in the ratio (71:12:17, v/v/v). This sample–ampholyte mixture was prepared in 0.1% aqueous HPMC solution, containing 1% TEMED and 0.085 *M* CAPS, 6% saccharose as solubilisers. The applied voltage of 425 V/cm which was optimised after studying the variation of voltage between 400 and 600 V/cm, simultaneously focused and mobilised the glycoforms of rgp 160.

3. Results and discussion

Both one-step and two-step cIEF approaches could be used for the analysis of rgp 160 glycoforms. However, we selected the one-step technique since the two-step one had longer analysis times. In this method, the pH gradient is established only in the shorter section of the capillary located after the detection window. Simultaneously to the focusing step the focused protein zones are mobilised past the detection window by means of the residual EOF towards the cathode, the most basic p*I* markers migrating first. Addition of TEMED which acts as a basic blocker occupying the blind end of the capillary between the inlet and the detector is necessary to prevent focusing of proteins before the detection window (Fig. 1). A PVA coated capillary exhibiting a residual EOF was selected for the one-step cIEF studies as several authors [28,29] have shown that uncoated capillaries have the disadvantage that the EOF is too high and that its magnitude is a function of the pH resulting in markedly curved p*I*–migration

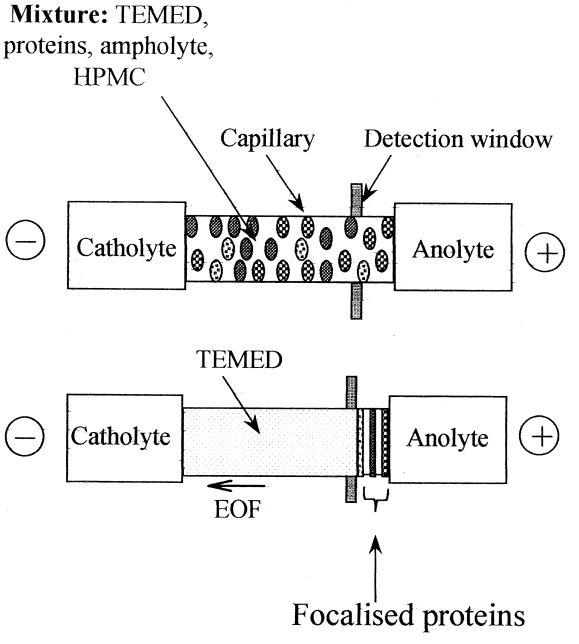

Fig. 1. One-step cIEF technique: TEMED was used as a basic blocker on the detector-distal-capillary segment, residual EOF mobilised proteins simultaneously with the focusing past the detector window towards the cathode.

times dependence. However, even with a PVA coated capillary, addition of HPMC was necessary to control the reproducibility from run to run. Different concentrations of this polymer from 0.1 to 0.4% (w/v) were tested. Migration times increased with increasing the HPMC concentration, broad peaks and capillaries clogging occurred for concentrations above 0.4%. The best compromise between analysis time and resolution between two close p*I* markers (carbonic anhydrase p*I* 5.9 and β-lactoglobulin p*I* 5.1) was attained using 0.1% HPMC. Replacement of HPMC by PEG did not improve the results.

Concentrations of TEMED in the range 1 to 6% (v/v) were then investigated for the separation of five p*I* standard markers in order to ascertain that the glycoprotein of interest focused in the right zone of the capillary between the outlet and the detector. We found little effect on either resolution or overall elution time for low concentration of TEMED. When the TEMED concentration was increased over 2%, long migration times and loss of the most acidic p*I* markers were observed. Use of 1% TEMED was sufficient to detect both the acidic and the most basic p*I* markers. In the same manner, the influence of the concentration of phosphoric acid in the anolyte in the range of 10 to 100 m*M* was studied. Phosphoric acid (100 m*M*) was found to give the best results in terms of resolution and analysis time.

During initial optimisation of the one-step cIEF method, ampholytes covering a wide range (pH 3–10) were employed. CAPS was used as a solubiliser since zwitterions have been advocated to solve the problem of poor protein solubility [41]. Based on the comparison of the migration times of p*I* internal standards, the p*I* of glycoforms of rgp 160 were estimated between pH 2.75 and 5.1.

We have compared different commercially available carrier ampholytes such as pH 3–10 ampholytes and pH 3.5–10 ampholines on the cIEF separation of the rgp 160 glycoforms since the distribution of the low-molecular-mass amphoteric substances in the pH gradient and their composition may vary. In contrast to previous observations [34,35,40], we found that the glycoform patterns were quite similar whatever the ampholyte employed. Approximately four peaks were partially separated with ampholine 3.5–10 (Fig. 2A), a little more with ampholyte 3–10 which separated more acidic glycoforms due to its larger range of pH. However, this latter ampholyte gave a higher signal-to-noise ratio as already observed by Chen et al. [34]. To further improve the separation, we have studied mixtures of wide and narrow range ampholytes. The best separation of rgp 160 glycoforms was obtained when the wide pH range 3.5–10 ampholytes was mixed with ampholytes covering a narrower pH range 3.5–5 and/or pH 5–8. The ratio giving optimal resolution and separation was determined to be a (71:12:17, v/v/v) mix of pH 3.5–5, pH 5–8 and pH 3.5–10 ampholytes. The ternary mixture (Fig. 2C) or the (50:50, v/v) mixture (Fig. 2B) of pH 3.5–10 and pH 3.5–5 ampholytes spread the glycoforms to a greater extent than the wide range pH 3.5–10 alone. A spike appeared in each electropherogram of rgp 160 indicating that partial precipitation of the glycoprotein still occurred. The binary or ternary mixtures of ampholytes mainly separated rgp 160 into several major and minor peaks (14 and 11 peaks, respectively). Nevertheless, the glycoforms were not sufficiently resolved. An optimisation of the total percentage of ampholytes in the range 2.5–10% (v/v) has been carried out as we anticipated that the increase of this concentration should favour the protein solubilisation. A 5% of total ampholytes was adopted for the separation of glycoforms of rgp 160 (Fig. 3A) as a slightly better resolution was observed. Concentrations over 5% were not satisfactory due to a higher signal-to-noise ratio. However, the presence of spikes in the electropherogram of the rgp 160 revealed that the precipitation phenomenon was not fully overcome. One of the strategies to minimise protein precipitation is to reduce the concentration of rgp 160. We observed only a few peaks when the concentration of rgp 160 was below 0.2 μg/μl and interestingly no or very broad small peaks when the concentration was over 0.9 μg/μl causing the very late migration of broad peaks probably representing aggregated forms of the rgp 160 (data not shown). We observed that the rgp 160 concentration compatible with the cIEF separation corresponded to the very narrow range of concentration of 0.2 to 0.9 μg/μl.

Other attempts to reduce precipitation by supplementing the sample–ampholyte mixture with various additives such as ethylene glycol or PEG, zwitterions (MES or tricine) and non-ionic surfactants (Nonidet P-40, reduced Triton X-100) failed (Fig. 3B). None

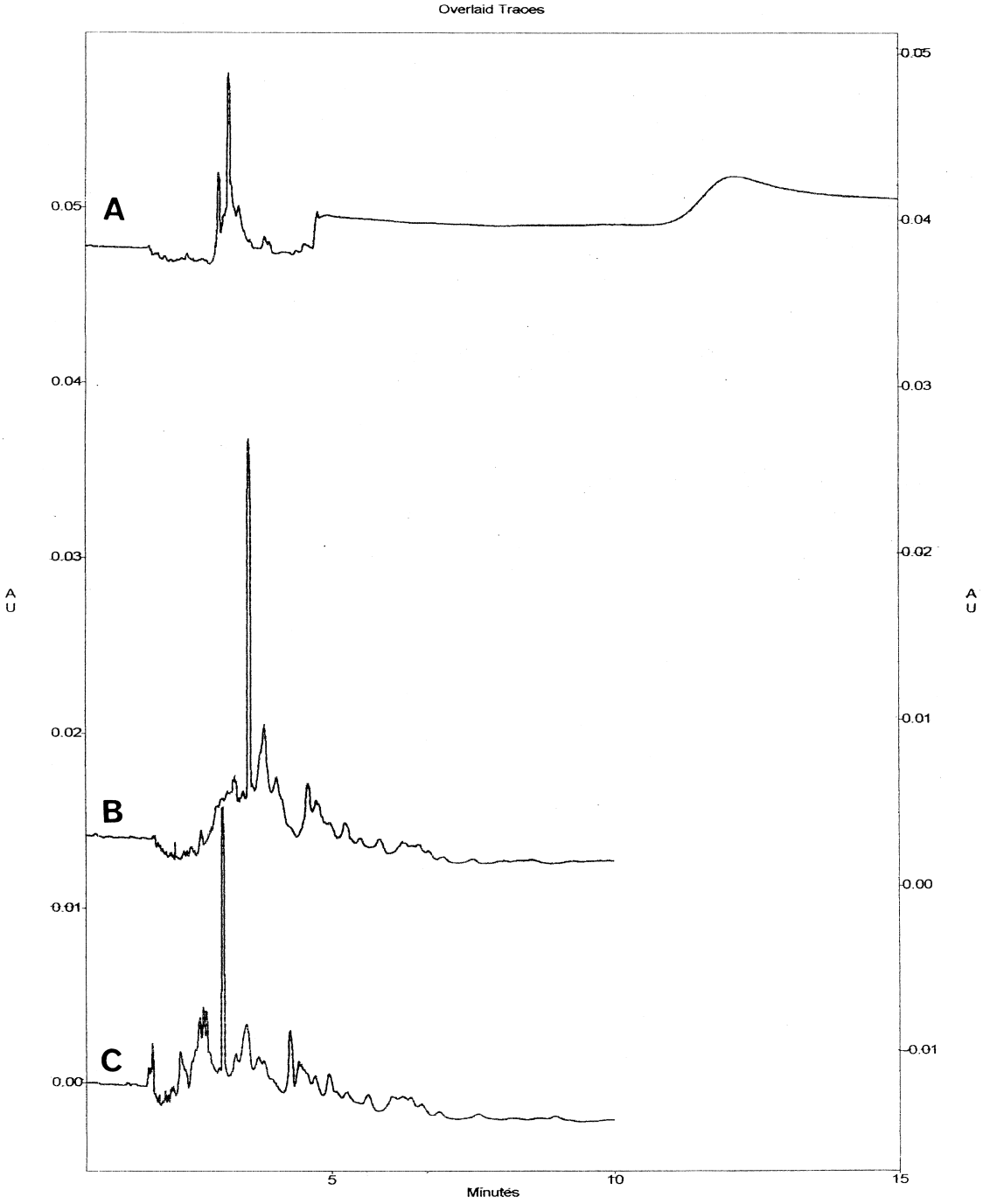

Fig. 2. Effect of the ratio pH 3.5–5, pH 5–8 and pH 3.5–10 ampholytes on the one-step separation of rgp 160 glycoforms: (A) pH 3.5–10 ampholines, (B) (50:50, v/v) mixture of pH 3.5–5 ampholines and pH 3.5–10 ampholytes, (C) mixture of pH 3.5–5 ampholines, pH 5–8 ampholines and pH 3.5–10 ampholytes in the ratio (71:12:17, v/v/v). Conditions: PVA coated capillary: 47 cm total length×50 μm I.D. Carrier ampholyte solution: 5% ampholytes, 1% TEMED, 0.15 *M* CAPS in 0.1% HPMC. Anolyte: 100 m*M* H_3PO_4, catholyte: 20 m*M* NaOH. Focusing and mobilisation at 20 kV at reversed polarity. Concentration of rgp 160: 0.3–0.8 μg/μl.

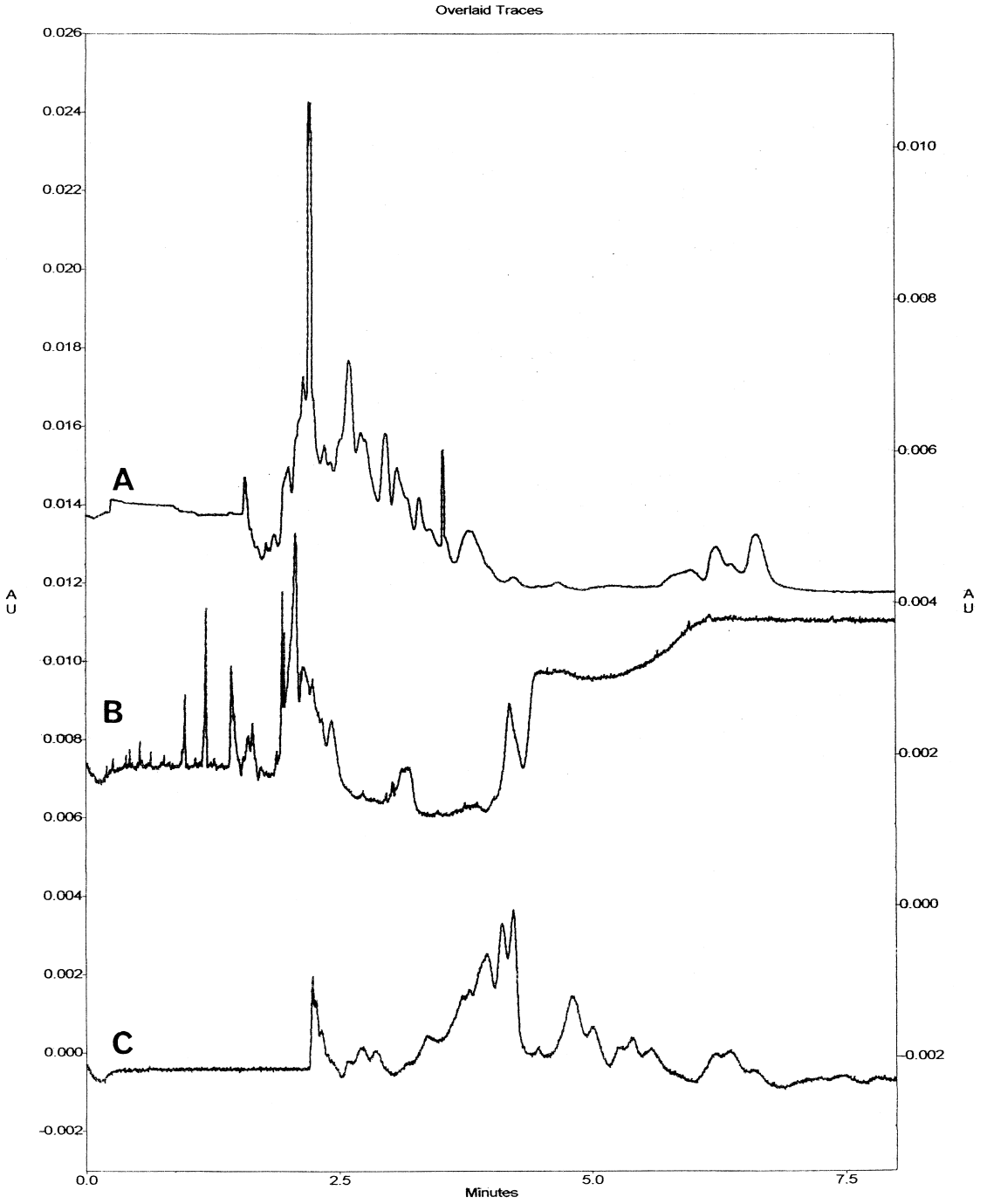

Fig. 3. Effect of different additives used on the glycoform profile of rgp 160: (A) 0.15 *M* CAPS, (B) 4% reduced Triton X-100 and (C) 4 *M* urea. Conditions: PVA coated capillary. Carrier ampholyte solution: 5% of pH 3.5–5 ampholines, pH 5–8 ampholines and pH 3.5–10 ampholytes in the ratio (71:12:17, v/v/v), 1% TEMED in 0.1% HPMC. Other conditions as in Fig. 2.

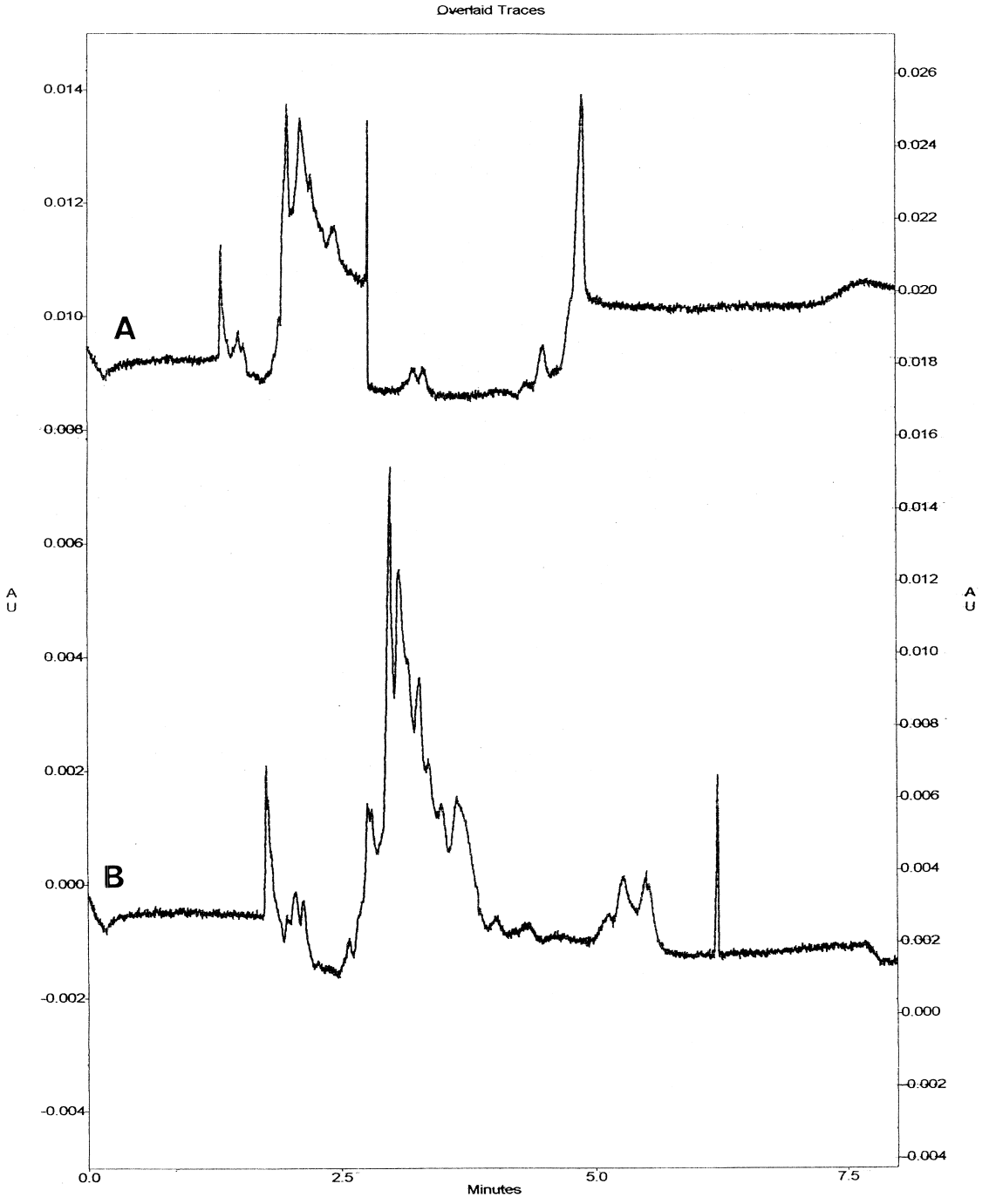

Fig. 4. Synergetic effect of the combination of zwiterrions and sugars as additives for the solubilisation of rgp 160: (A) 0.085 *M* CAPS, (B) 0.085 *M* CAPS and 6% saccharose. Conditions: PVA coated capillary. Concentration of rgp 160: 1.7 μg/μl. Other conditions as in Fig. 3.

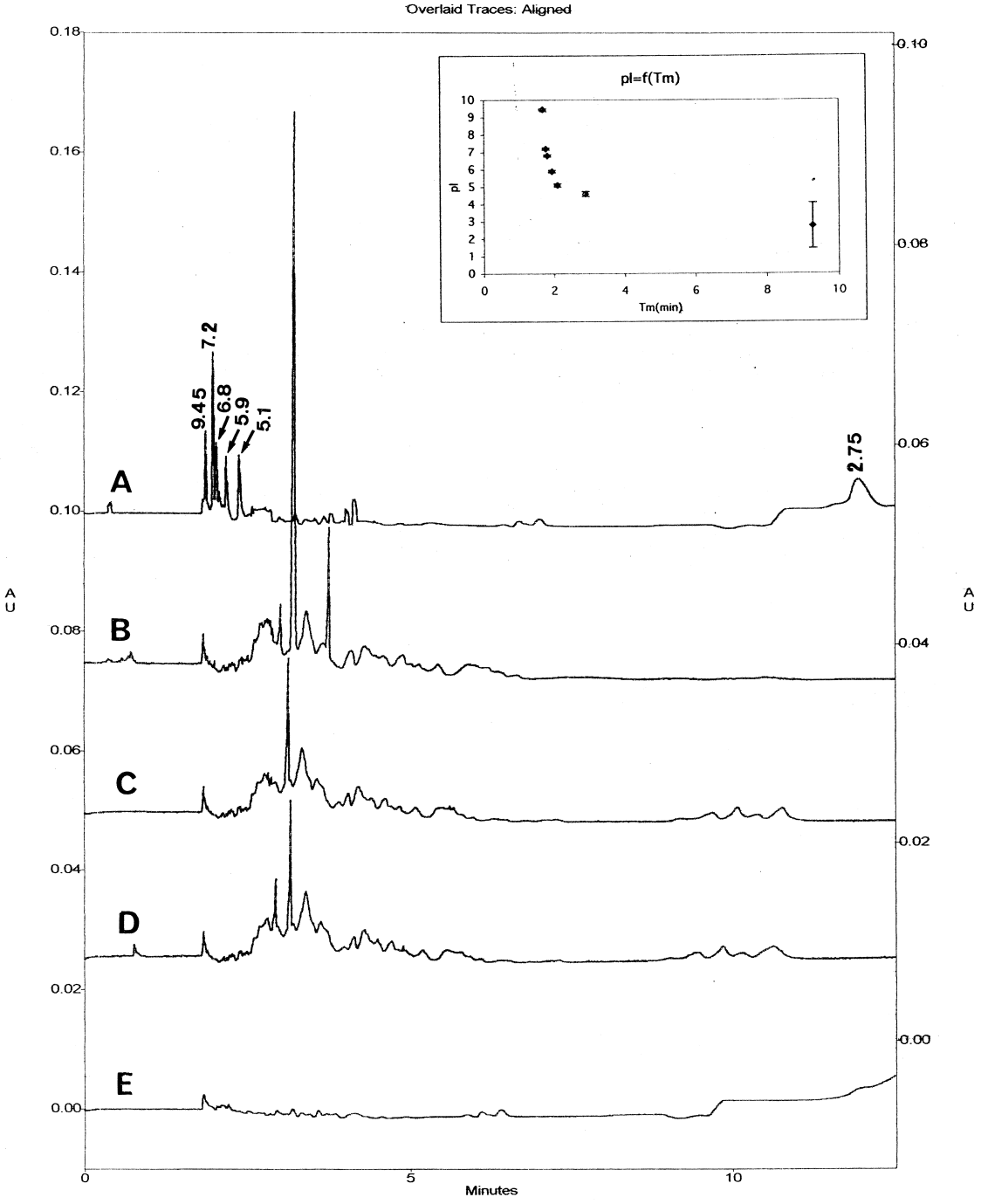

Fig. 5. (A) One-step cIEF electropherogram of the protein standards. (B–D) Reproducibility of the separation of rgp 160 by the one-step cIEF. (E) Background electropherogram. Inset: Calibration curve (p*I* vs. migration times) made from the mean of six analyses of the protein standards, bars indicate the corresponding SD. Conditions: PVA coated capillary. Carrier ampholyte solution: 5% of pH 3.5–5 ampholines, pH 5–8 ampholines and pH 3.5–10 ampholytes in the ratio (71:12:17, v/v/v), 1% TEMED, 0.085 *M* CAPS, 6% saccharose in 0.1% HPMC. Concentration: 0.3–0.8 μg/μl of rgp 160 and 0.01–0.4 μg/μl of p*I* standard markers. Other conditions as in Fig. 3.

of them improve the resolution attained using CAPS. Native cIEF is generally successful for proteins below a molecular mass of 150 000 with high solubility at a pH close to its p*I*. For other proteins, denaturing cIEF in the presence of urea at high concentrations (2–8 *M*) has been suggested as a viable alternative [20]. Urea (4 *M*) allowed a better solubilisation of rgp 160 than the other solubiliser agents tested since no spike appeared in the electropherogram (Fig. 3C). However, this well-known protein solubiliser unfolds proteins and under these conditions, the p*I* scale obtained for rgp 160 could not be compared with that under native cIEF. For this reason, this condition was therefore not retained. The class of sugars (saccharose and sorbitol) turned out to be quite ineffective. In the hope of a synergetic effect between different solubilisers/additives, we have investigated mixtures of sugars and zwiterrions. To greatly differentiate the effect of CAPS alone or CAPS in combination with sugar on protein solubilisation, we have chosen to work with a concentration of rgp 160 above the optimal range of 0.2–0.9 μg/μl where the precipitation phenomenon is assumed to be more pronounced. Good resolutions and separation patterns were finally obtained with mixtures of 0.085 *M* CAPS and 6% saccharose. A highly concentrated rgp 160 sample showed effectively a partial separation in the presence of CAPS and saccharose (Fig. 4B) whereas CAPS alone could obviously not solubilise rgp 160 at this concentration since the profile obtained presented a poor separation and a spike indicating the non-solubilisation of rgp 160 (Fig. 4A). Interestingly, the zwitterion taurine replacing CAPS in this mixture gave a similar profile but with less peaks.

The profiles obtained under optimal conditions [1% TEMED, 0.1% HPMC, (71:12:17, v/v/v) mix of 5% ampholytes pH range 3.5–5, 5–8 and 3–10, 0.085 *M* CAPS and 6% saccharose] for five standard markers with p*I* values ranging from 2.75 to 9.45 and for rgp 160 are shown in Fig. 5. The background electropherogram showed some minor peaks not interfering with the rgp 160 profiles. The migration times of the p*I* standards were found to be affected by the presence of rgp 160 showing decreased migration times and closer peaks (data not shown). This observation was more marked for the most acidic p*I* standard. The migration time shift observed for the CCK flanking peptide (p*I* 2.75) attained up to 7 min. This feature might have resulted from the presence of residual salts in the glycoprotein sample altering the pH gradient generated on focusing. Interaction between rgp 160 and the markers may also be responsible for a different behaviour when rgp 160 was added to the p*I* marker standards. A similar result has been previously reported by two groups [21,35].

The intra-day reproducibility of migration times and relative peak areas for the five p*I* standards has been calculated from six replicate injections (Table 1). The precision of this method was very satisfactory with migration times being less than 0.6% RSD (relative standard deviation) except for the CCK peptide presenting a RSD of 1.9%. This low precision obtained for CCK migration time is probably due to its low p*I* which is outside the gradient pH generated by the ampholytes mixture. This excellent migration time reproducibility for the other p*I* markers was attributed to the beneficial effect of the addition of HPMC into the sample–ampholyte mix-

Table 1
Intra-day reproducibility of migration times and relative areas from six replicate injections of protein standards (with p*I* ranging from 2.75 to 9.45) using a PVA coated capillary

Standards	p*I*	Migration time		Relative area
		Average ($n = 6$)	RSD ($n = 6$)	RSD ($n = 6$)
Ribonuclease A	9.45	1.88	0.54	1.61
Myoglobin	7.2	1.99	0.56	6.00
Myoglobin	6.8	2.04	0.53	14.52
Carbonic anhydrase II	5.9	2.19	0.52	2.73
β-Lactoglobulin	5.1	2.40	0.57	4.26
CCK-flanking peptide	2.75	12.13	1.88	2.62

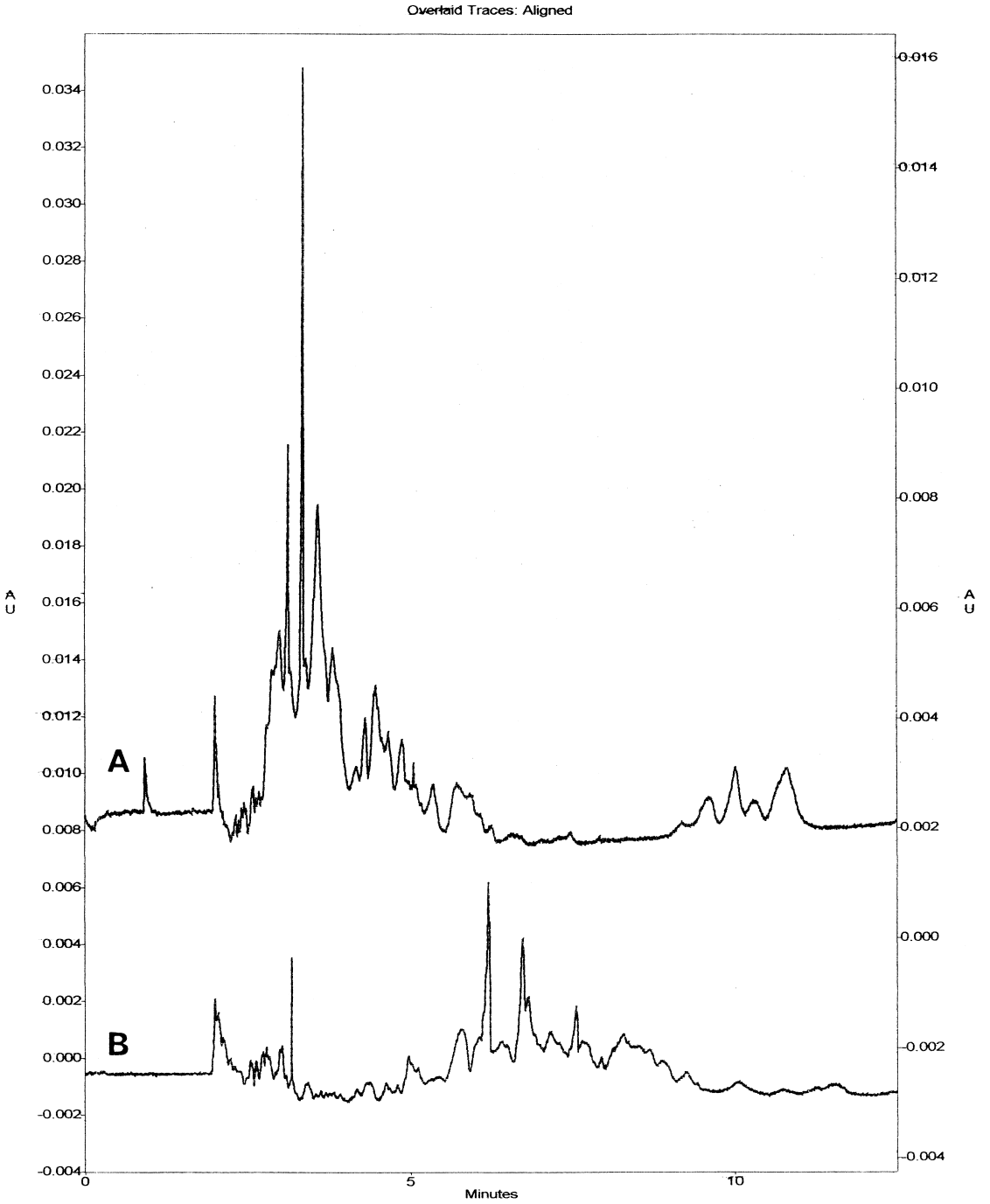

Fig. 6. Comparison of two commercially available capillary coatings from Beckman: (A) PVA, (B) PAA for the one-step cIEF of rgp 160 glycoforms. Conditions as in Fig. 5.

ture. Nevertheless, reproducibility of relative areas (<6% RSD) was relatively acceptable except for Mb (p*I* 6.8) representing a minor peak. Although the reproducibility of rgp 160 separation depicted in Fig. 5B, C and D was then confirmed, spikes appeared sometimes indicating that the protein insolubility phenomenon was not completely resolved.

The inset of Fig. 5 displays the plot of p*I* vs. migration times for the p*I* markers, which showed a non-linear relationship. Adequate linearity of the p*I* calibration curve could then be yielded only for short p*I* ranges (pH 5.1–9.45 and 2.75–5.1) where the calibration curve could be approximated by a straight line. Contrary to our expectation based on the p*I* value predicted for the rgp 160s MN/LAI polypeptide backbone chain (around 8.4), the p*I* of rgp 160s MN/LAI was determined to be in the range of pH 4.00 to 4.95 by cIEF. In addition, Stein et al. [45] have determined the p*I* of the mature gp 160 envelope precursor in the pH range of 7.0 to greater than 7.5 using two-dimensional gel electrophoresis under denaturant conditions in the presence of SDS. In another work, Hu et al. [46] reported that the envelope glycoprotein of HIV-1 gp 120 is heavily sialylated and electrofocussed to an acidic pH range. The unexpected acidic p*I* values we obtained could be explained firstly by the high sialylation degree of oligosaccharides derived from rgp 160s MN/LAI [15] that could be also associated with moieties other than complex *N*-linked oligosaccharides (e.g. *O*-glycans) [45] and secondly by the non-denaturing conditions we employed for its separation.

Although intra-day reproducibilities were quite satisfactory, we observed that the stability of the PVA coating was quite limited as, after an average of 100 runs, a significant shift of the migration times was observed for the rgp 160 as well as for the p*I* markers. This shift was attributed to a gradual increase of the EOF from run to run which was associated with a degradation of the coating from exposure to the basic catholyte and TEMED. The same observation was made immediately after using urea in the analysed mixtures. The problem of coating stability in cIEF, related to the exposure of the coating to strong acid and base as anolytes and catholytes, respectively, has been raised by several authors [35,47–49]. We have therefore compared the PVA coated capillary with a second coated capillary: PAA for their resolving capabilities and their stability using the same method. Fig. 6 compares the separation of rgp 160 obtained on a PVA coated capillary and on a PAA coated capillary. Both capillaries gave equivalent resolution but the PAA capillary showed longer migration times and a profile more extended compared to that obtained with the PVA capillary, indicating that the PVA coated capillary exhibits a higher residual EOF. As a general trend, intra-day reproducibility ($n = 6$) of migration times using the PAA capillary was much lower (RSD from 3.8 to 9.6%) than that obtained with the PVA capillary (RSD from 0.5 to 1.9%) (Table 2). Long term stability of these coatings was also compared by measuring the migration times of the protein markers for the first run and after 40 and 60 runs. RSD values obtained for migration times were 5–10-fold higher with the PAA capillary (average RSD of 3.19% for the PVA capillary and 28.32% for the PAA capillary), indicating that not only the intra-day reproducibility but also the long term stability were lower in the case of the PAA capillary. Interestingly,

Table 2
Intra-day reproducibility of migration times and relative areas from six replicate injections of protein standards (with p*I* ranging from 4.6 to 9.45) using a PAA coated capillary

Standards	p*I*	Migration time		Relative area
		Average ($n = 6$)	RSD ($n = 6$)	RSD ($n = 6$)
Ribonuclease A	9.45	3.27	9.57	9.34
Myoglobin	7.2	3.48	9.45	10.18
Myoglobin	6.8	3.65	8.87	3.79
Carbonic anhydrase II	5.9	4.01	8.23	7.78
β-Lactoglobulin	5.1	4.57	7.97	3.69
Trypsin inhibitor	2.75	7.41	3.82	12.85

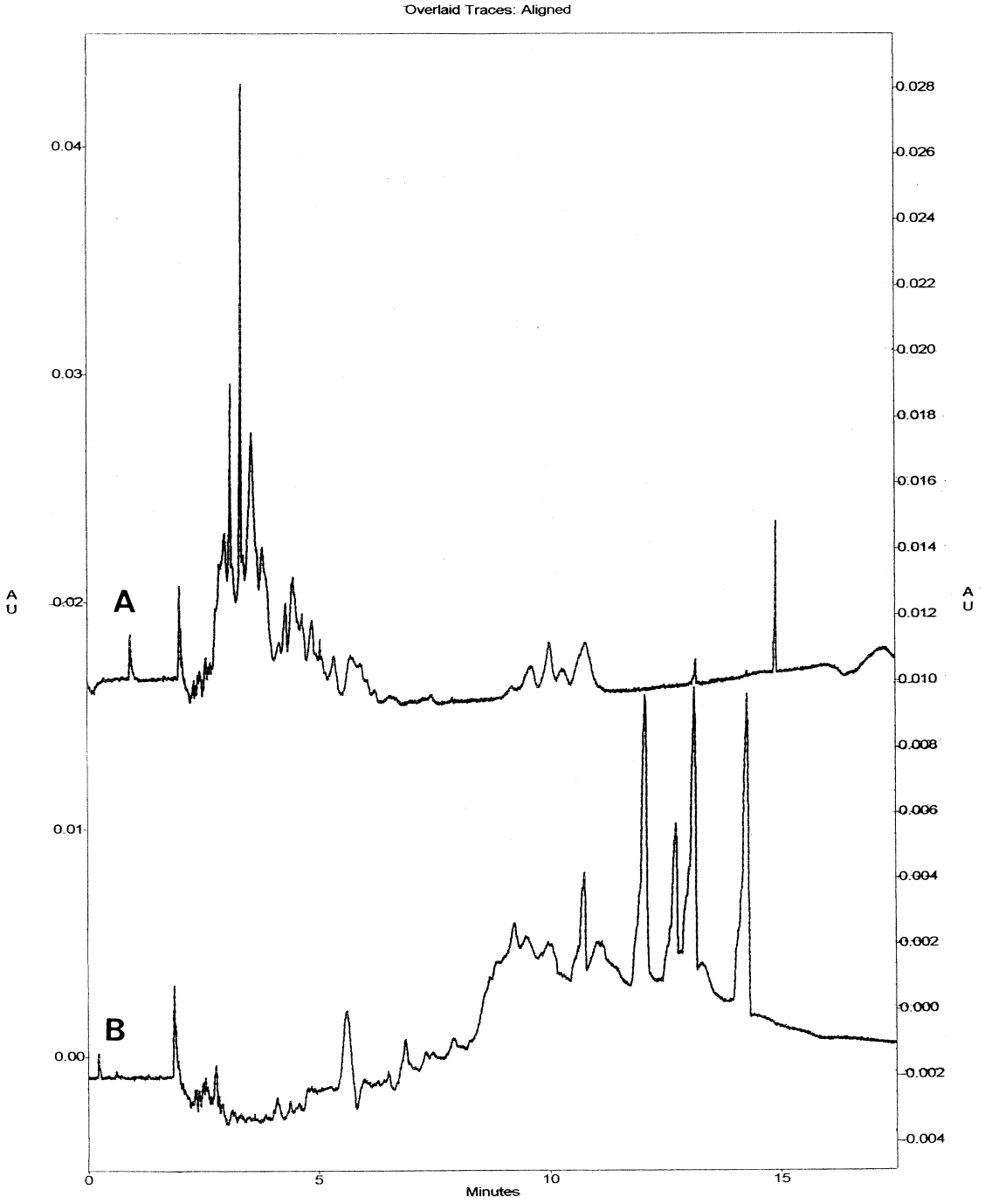

Fig. 7. Comparison glycoform profile of rgp 160 from two sub-populations of the virus HIV-1: (A) clade B, (B) clade E. Conditions as in Fig. 5.

migration times of the most acidic p*I* markers appeared to be more reproducible than the basic ones with the PAA capillary and the inverse tendency was observed in the PVA capillary. In addition, we observed for each marker a constant difference between the migration time obtained with these two capillaries except for the most acidic standard for which this difference was lower. This was explained by the low EOF at acidic pH attenuating the differences between the two capillaries. Moreover, we observed for the PAA capillary the same decoating phenomenon following the use of urea than for the PVA capillary. One alternative to improve this coating stability toward hydrolysis has been suggested by Gelfi et al. [50] who reported that the Si–C–polyacrylamide capillaries were more stable than the Si–O–polyacrylamide capillaries.

Attempts to employ uncoated capillaries with the addition of 0.1 to 1% HPMC or 0.5% PVA in the sample to reduce and control EOF as suggested by several authors [28,29] remained unsuccessful. In spite of the presence of these polymeric solutions, the EOF remained too high leading to a poor resolution of rgp 160 glycoforms.

As an application the method was used to compare the rgp 160 of two sub-populations of the virus HIV-1 called clades B and E (Fig. 7). Both profiles of clade B and clade E differed in terms of number of peaks and migration times, suggesting that clade E exhibits not only a lower microheterogeneity than clade B but also has more acidic glycoforms. These findings are in accordance with results we obtained recently from the rgp 160 derived *N*-oligosaccharide mapping (manuscript in preparation) and showing that rgp 160 of clade E bore complex-type structures with a higher degree of sialylation. However, we cannot exclude the possibility that these differences are also due to a different amino acid sequence of the rgp 160 arising from the two clades. Moreover, even if additional work is needed to fully interpret the differences observed between these two clades in terms of glycosylation, the cIEF appears a valuable method, as a first stage, to point out differences in the glycoform pattern of two close glycoproteins.

4. Conclusion

In this report, we have shown the potentialities (rapidity and precision) of the one-step cIEF approach in the analysis of the microheterogeneity of recombinant glycoproteins and demonstrated its applicability for the analysis of rgp 160. This method did not allow quantitation of rgp 160 glycoforms but is suitable for comparison of two recombinant glycoproteins. This ability of the method to differentiate two sub-populations of virus HIV-1 originating from two continents was clearly demonstrated and could be very useful considering that five different clades of this virus exist all over the world. A key to the successful separation of rgp 160 glycoforms would be to further increase its solubility properties in order to analyse a high enough sample concentration. Reproducibility of the method appears to be greatly dependant on the stability of the coating and is therefore influenced by the capillary age. Meanwhile, the reproducibility could be further improved by testing other kinds of coated capillaries or by compensating the increase of the EOF associated with a degradation of the PVA coating.

Although the one-step cIEF method using EOF mobilisation is rapid and globally reproducible, it is expected that the two-step cIEF technique using capillaries with zero EOF and pH stable coatings could improve the reproducibility of peak migration times. Moreover, this second approach provides a linear relationship between p*I* and migration times, avoiding the use of internal standards for the determination of the p*I*. The performance of such a two-step approach for rgp 160 is currently being investigated.

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