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Glycosylation

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Contents

1. ANALYSIS OF GLYCANS OF RECOMBINANT GLYCOPROTEINS

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1. Introduction

An important aspect of recombinant protein analysis is the consideration of post translational modifications. This review will focus on the analysis of glycosyla-tion, which is one such major modification. A number of studies have shown that glycosylation depends on both the cell line used for the culture and other factors in the culture medium (Doyle, de la Canal et al., 1986); (Page, Killian et al., 1990) (Patel, Parekh et al., 1992; Borys, Linzer et al., 1993) (Gawlitzek, Valley et al., 1995) (Andersen, Bridges et al., 2000; Yang and Butler, 2000). It is important to consider these factors and also to be able to monitor glycosylation as this may affect several properties of the glycoprotein under production, most significantly the half life in the circulation, a factor that is an important aspect in considering the pharmokinetics of recombinant glycoproteins (Baynes and Wold, 1976); (Elbein, 1991; Goochee, 1992; Kobata, 1992; Dwek, 1995; Rice, Chiu et al., 1995) such as the immunoglobulins ((Elbein, 1991; Kunkel, Jan et al., 1998; Tang, Nesta et al., 1999).

The study of the glycan chains (oligosaccharides) attached to glycoproteins presents a number of analytical problems that generally makes their analysis more difficult than that of the peptides to which they are attached. Predominant among these problems are the branched nature of many carbohydrates and the ability of constituent monosaccharide units to form bonds to several positions on the adjacent unit. The following analytical techniques have all been applied to the study of glycans.

1. Nuclear magnetic resonance (NMR)
2. Analysis by high pH anion exchange chromatography (HPAEC)
3. Analysis by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)
4. Analysis by capillary electrophoresis (CE)
5. Analysis by gel permeation chromatography (GPC)
6. Analysis by high-performance liquid chromatography (HPLC) following fluorescent labeling
7. Analysis by mass spectrometry (MS)

M. Al-Rubeai (ed.), Cell Engineering, 1-60.

Each technique has its own merits and all have been successfully employed for the characterisation of recombinant glycoproteins. The choice of technique is, therefore, dictated by a number of factors including level of information required, available expertise and budget. The speed of analysis and interpretation of data may also be considerations.

Definitive characterisation has traditionally been performed by NMR (Anderson, Atkinson et al., 1985); (Anderson, Atkinson et al., 1985); (De Beer, Van Zuylen et al., 1996). However, this technique is not generally applicable to analysis in a routine manner as it requires relatively large amounts of material (in the milligram range). In addition, access to the sophisticated equipment and the expertise required for the interpretation of data mean that the technique is only available to specialised dedicated laboratories. Another complex technique which has also been applied extensively to studies of glycan structure is that of mass spectrometry in various forms, several of which are discussed below. Electrophoretic techniques have several advantages in terms of speed and generally do not require sophisticated instrumentation. The most widely used are those employing polyacrylamide gels or capillary electrophoresis. Chromatographic techniques offer a number of advantages and various forms have been widely used. In particular, the use of fluorescent labels such as 2-aminobenza-mide (2-AB) and HPLC column systems calibrated with standards allows the assignment of structures and comparative profiling. In combination with exogly-cosidase digestion and mass spectrometry, complete analysis of complex glycans may be achieved. Monosaccharide analysis is a traditional means of analysis and is still widely used in quality control although it is now becoming replaced by methods that give more information on glycan structure.

2. Analysis Of Intact Glycoproteins

2.1. ANALYSIS BY CAPILLARY ELECTROPHORESIS

The emergence of capillary electrophoresis (CE) with the various modes in which separation can be performed has brought new possibilities in the field of microheterogeneity of glycoproteins evaluations. CE method, combining the advantages of high efficiency and resolution, accurate quantification, short analysis time and automation, has gained interest not only in lot-to-lot routine control of glycoproteins produced by DNA technology, but also in process monitoring, purity assessment and product quality evaluation.

In this chapter, applications of recombinant glycoprotein analyses with the aim to separate either the glycoforms of the intact glycoprotein or oligosaccharides derived from glycoproteins will be described. A great number of publications have reviewed the potential of this technique for the analysis of recombinant glycoproteins (Chiesa, Oefner et al., 1995; El Rassi and Mechref, 1996; Taverna, Tran et al., 1998).

2.1.1. Glycoform Analysis

This part will consider the main strategies for analysing the glycoforms of the intact glycoproteins by means of the most employed modes afforded by CE. This approach

represents the most straightforward method to establish a pattern of glycoprotein heterogeneity; and information on the identity, the heterogeneity, the purity of the glycoprotein are readily obtained.

Due to their high resolving power, capillary zone electrophoresis (CZE) and capillary isoelectric focusing (CIEF) are the most employed for sensitive and rapid screening techniques for recombinant glycoproteins. Owing to their ease of automation and their selectivity which facilitate the development of validatable routine analysis, CZE and CIEF appear well suited for the quality control environment.

Generally, when the analyses of an unknown glycoprotein is required, the first and the simplest mode that should be tried to separate the different glycoforms is CZE in uncoated capillaries, which can be carried out with a reasonable number of parameters to optimize. CIEF can then be employed either to further improve the resolution or to achieve separations that could not be possible using CZE mode. This mode is particularly suitable for separation of glycoproteins bearing complex type oligosaccharides, or which glycoforms exhibit different degree of sialylation, sulfatation or phosphorylation. The practical application of different microscale techniques of CE for analyzing the intact recombinant glycoproteins (Taverna *et al.*, 2001).

2.1.2. Capillary Zone Electrophoresis (CZE)

CZE separation is based on charge-to-mass ratio. In the case of glycoforms, CZE is well suited to the separation of variants with different glycosylation degrees and different number of sialic acid residues. It has been shown that neutral glycoforms can also be separated by careful selection of the separation conditions (using the complexation of sugars with borate).

It is therefore recommended to firstly use a wide pH range to ensure that at least one peak can be visualized, improvement of the glycoform separation is then accomplished by testing pHs closer to the isoelectric point of the protein where differences in their charge to mass ratio is more pronounced. When glycoforms differ by only slight variations in their glycosylation, reduction of the electroosmotic flow (by adding alkylamines to the separation buffer) may be necessary to achieve a complete resolution of the glycoforms. Watson and Yao (Watson and Yao, 1993) have first reported the well-known separation of the glycoforms of recombinant human erythropoietin (rHuEPO) into six well resolved peaks using an uncoated silica-fused capillary in a tricine buffer (pH 6.2) with the addition of 2.5 mM 1,4-diaminobutane (DAB) and 7M urea. Sialic acid residues are negatively charged at neutral pH values and thus, glycoforms eluted in a predictable manner in order of increasing numbers of sialic acid residues.

Although CZE has been the most studied mode for glycoforms separation, the major problem encountered is the adsorption of glycoproteins to the capillary wall. In this case, permanent coated or dynamically coated capillaries should be preferred (the latter ones offering the advantages to be cheaper, more stable and more compatible with the diode array detection). Several papers have also reported the use of neutral polymers like cellulose derivatives (*e.g.* methyl cellulose, hydroxyethyl cellulose (HEC), hydroxypropylmethyl cellulose (HPMC)) to pre-vent protein adsorption by dynamically coating the silica wall. The modified cellulose are generally used at very low

concentrations. Phosphate ions are believed to strongly bind to the capillary surface converting acidic silanols to protonated silica-phosphate complex. In this case, capillaries are also called phosphate deactivated fused-silica.

Thus, CZE may be used for on-line analysis to monitor bioproduction of pharmaceutical glycoproteins at different stages of the purification or production process such as cultivation step, downstream process. CZE may also contribute to assess the recombinant glycoprotein identity, its purity, heterogeneity, quantity and stability.

2.1.3. Process Control

Reif *et al.* (Reif and Freitag, 1995) have reported the control of the cultivation process of recombinant antithrombin III (r-AT III). The downstream process was monitored by CZE using 50 mM phosphate buffer with 0.1% hydroxypropylmethylcellulose (HPMC) (pH 2.0) at 20°C, and an uncoated capillary under voltage ramping. The detection limit was found to be 50µg/ml using detection at 200nm increasing the sensitivity. One advantage of using CZE is that analysis time of 10 min is regarded as acceptable, especially as the down-streamm process takes several hours.

Somerville *et al.* (Somerville, Douglas et al., 1999) reported the CE separation of glycosylated and non-glycosylated recombinant human granulocyte macrophage colony stimulating factor (rHuGM-CSF) in 40 mM phosphate buffer, pH 2.5 and containing 0.03% HPMC. Glycosylated G-CSF eluted as a double peaks compared to the non-glycosylated recombinant human granulocyte colony stimulating factor (G-CSF) that eluted as a single and sharp peak. This technique enabled a detection limit of 10 µg/mL for the rHuGM-CSF. The doublet peaks could be due to the neuraminic acid residue present at the threonine site on the glycosylated molecule. This method has been used to reveal distinct differences between the bacculovirus expressed products and the comercially available glycosylated rHuGM-CSF, since bacculovirus expressed G-CSF gave a peak shape similar to that obtained for the non-glycosylated form although it is glycosylated. The difference in the glycosylation pathway between insect and mammalian cell explained the absence of the doublet peaks. These results have confirmed other reports indicating that insect cells are unable to process complex carbohydrate structures.

Using the phosphate deactivated fused-silica capillary method, an interesting application of CZE method to detect the effect of changes in the fermentation conditions on recombinant human growth hormone (rhGH) production from *E. Coli* has been reported. The rhGH and its variants could be resolved in less than 20 min from very crude mixtures of *E. Coli* using a 250 mM phosphate (pH 6.8)-1% (v/v) propylene glycol buffer whereas three different HPLC separations were required to detect the natural rhGH and its variants (McNerney, Watson et al., 1996).

2.1.4. Purity Testing

Pedersen *et al.* (Pedersen, Andersen et al., 1993) have studied microheterogeneity of the proteinase A for the evaluation of product purity and also of the suitability of the host organism. The CZE analysis of the proteinase A and the variant glycoform was performed, using an untreated silica capillary with 100 mM acetate-phosphate buffer

(pH 3.2). Both molecules resolved into three peaks that probably correspond to charge heterogeneities atributable to differences in the phosphorylation level of the carbohydrate group at Asn68. This study revealed that approximately 70% of the product was native proteinase A presenting two glycosylation sites Asn68 and Asn269, whereas the remaining 30% was a proteinase A variant glycoform lacking the carbohydrate moiety at Asn269.

2.1.5. Batch-to- Batch Consistency

Hoffstetter-Kuhn *et al.* (Hoffstetter-Kuhn, Alt et al., 1996) have described the use of CZE based on complex formation of borate with carbohydrates in a borate buffer at pH 9.4 to monitor batch-to-batch consistency of monoclonal antibody (mAb). It was found that lower temperatures, higher borate concentrations and higher pH values improved the separation by stabilizing the complex, giving three peaks detected by UV absorbance at 200 nm. Firstly, linearity of the peak areas was measured up to a protein concentration of 0.1% w/v, with correlation coefficients better than 0.999. On the other hand, the separation method was validated for reproducibility (n=6) as the individual peaks were quantified with fairly good precision with relative standard deviations of the peak area ranging from 5.5% to 7.3% (5.1% for peak 3, 5.5% for peak 2 and 7.3% for peak 1). Electropherograms of different batches of the mAb showed two profiles quite similar while the third is markedly different. Although all three peaks were detected in the latter profile, the relative peak areas deviated significantly. This technique was therefore useful for stability testing of galenical formulations.

2.1.6. Stability and Quantification

The precedent method described by Hoffstetter-Kuhn *et al.* (Hoffstetter-Kuhn, Alt et al., 1996) showed that separation profiles changed distinctly after antibody storage in glass vials for three months at different temperature. Pronounced degradation (only one of the initial three peaks could be detected clearly) was observed at 37°C and even more after storage at 37°C for six months. Other stability testing of the final product formulations such as recombinant human tumor necrosis factor beta (rHuTNF-β) (Yao *et al.*, 1995) (Yao, Loh et al., 1995) and immunoglobulin G (IgG) (Klyushnichenko and Kula, 1997) (Klyushnichenko and Kula, 1997) have been successfully reported using borate as CZE buffer.

Bietlot *et al.* (1997) (Bietlot and Girard, 1997) developed a CZE method for quantitative recombinant human erythropoietin (rHuEPO) determination in final drug formulations. Large amounts of human serum albumin (HSA) are generally added to rHuEPO as a protein excipient. A complete separation of the two proteins without affecting the resolution pattern of rHuEPO into several glycoform populations could be achieved only through the addition of 1 m*M* nickel chloride to a 200 m*M* phosphate buffer, pH 4 with a fused silica capillary. Indeed, metal ions are known to interact with (glyco)proteins, to affect their properties and conformation, and then to alter their electrophoretic mobility. Then, it appears that the presence of nickel ions in the buffer decreases the electrophoretic mobility of HSA. This method was linear over the concentration range of 0.03-1.92 mg/ml, with limits of detection and of quantitation of

0.01 and 0.03 mg/ml, respectively. The precision of the method was evaluated from intra- and inter-day triplicate injections of rhEPO standard solutions and formulations over four batches. This method was found to be useful for quantitative measurement rhEPO in formulations (components of within- and between-batch variances were less than 5%). In addition, this method was found to be useful to reflect variations in manufacturing processes. Clear differences emerged first in the number and relative amounts of the resolved glycoforms, and second in peak shape and relative proportions of the four largest peaks (see Figure 1).

2.1.7. Natural Versus Recombinant Glycoprotein Comparisons

CZE has been shown to be useful to compare interleukin-2 (IL-2) in its native state and Escherichia coli (E. Coli) derived recombinant IL-2 (rIL-2) (Knuver-Hopf and Mohr, 1995) Using a coated capillary and 100 mM pH 2.5 phosphate buffer at 10kV, CZE separation of natural interleukin-2 (nIL-2) shows three different forms (non-glycosylated, glycosylated-monosialylated and glycosylated-disialylated). CZE analysis of rIL-2 exhibited two peaks with approximately the same electrophoretic mobility as the first peak in the electropherogram of nIL-2 under the same conditions. The authors suggested that one of those two peaks represents a rIL-2 form with a conformation slightly modified. This work demonstrated the high selectivity of CZE for separation of proteins with a single charge difference.

The potential of CZE for the routine analytical characterisation of glycoform heterogeneity of rtPA has been demonstrated by Thorne, 1996. An excellent precision of the method was determined thanks to the high migration time reproducibilities (relative standard deviation is less than 0.2%) and full protein recovery (resulting of the

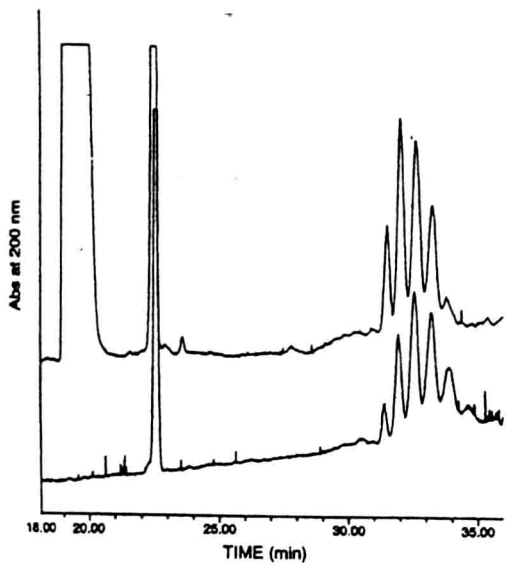

Figure 1. Comparison of the profile of rhEPO formulations from two manufacturers. Conditions: fused capillary 50μm×47cm, 200mM sodium phosphate buffer, pH 4.0, 1mM nickel chloride hexahydrate, 8kV, 20°C, 200nm.

addition of 0.01% (v/v) Tween 80 to the running buffer). These authors showed that CZE was a rugged technique using ω-amino acids buffer with PAA and PVA coated capillaries for the separation of rtPA variants.

2.1.8. Capillary Isoelectric Focusing (CIEF)

CIEF permits to analyze the charge heterogeneity of glycoforms on the basis of their degree of sialylation, phosphorylation and/or sulfatation.

The separation of charged analyte molecules takes place in a pH gradient created in a capillary by ampholyte mixtures under the influence of an electric field. Since the migration of charged molecules stop when their net charge is zero, they focalise in narrow zones in the region of pH corresponding to their isoelectric point (pI). A mobilization step is thus required to drive the focused zones past the detector window. Two CIEF techniques have been essentially developed:

In the two-step method, focusing and mobilization take place sequentially; the latter is achieved independently after the focusing step. Pressure mobilization is the simplest method. However if complete resolution of glycoforms is not attained, chemical mobilization may be preferred. Capillary with nearly no EOF have to be employed, using neutral coated capillaries such as polyacrylamide or polyvinyl alcohol coated capillaries.

In the one-step method, the components are mobilized simultaneously with the focusing, by means of the EOF which is maintained constant by the addition of polymers such as cellulose derivatives in buffers. Employing either uncoated or coated capillaries, faster separation are obtained using this approach. The stability of the coating is of prime concern as it will influence the velocity of the mobilization and thereby the reproducibility of the migration times.

For glycoproteins the pI depends not only on the number and type of charged amino acids of the protein backbone but also on the number of sialic acid residues of the glycan moiety. CIEF today offers a high resolving power (0.01-0.02 units of pH) and the advantages of automatization and rapidity. In addition, the concen-trating effect arising from the focusing step enables the detection of components present in small quantities. This mode of separation is particularly suitable for separation of glycoproteins bearing complex type oligosaccharides and exhibiting a good solubility in water. However, CIEF suffers from two main drawbacks including problems associated with reliability of pI markers, and with protein solubility.

The determination of pI values by CIEF is generally based on the comparison of migration times of the unknown peaks to those of pI standards added to the sample mixture (internal standards). Although protein with known pI may be used as pI markers, the main problem is their instability. Another approach consisted in the use of synthesized small molecular weight substituted aromatic aminophenols (Rodriguez-Diaz, 1997) synthetic (oligo)peptides (Shimura, Wang et al., 2000) with UV absorption which assure a pI reproducibility of 0.06%.

One of the main problem encountered in CIEF is precipitation of proteins at a pH close to their pI that may occur during the focalisation step, resulting in clogging the small-diameter capillaries used, in irreproducibility of migration times and peak area. A number has discussed about protein solubilization (Rabilloud, 1996); (Liu, 1996). In a

recent review, we have made up a list of different solubilizers employed for CIEF of glycoproteins (Taverna *et al.*, 2001). In addition reviewed in details the desirable properties of a carrier ampholyte and its influence on the protein solubility during the focusing step.

Another problem encountered with CIEF of glycoproteins is the presence of salts in the samples. These salts can affect the pH gradient and can have adverse effect on reproducibility (Grossman, Wilson et al., 1988). Thus, a small change of pI values of glycoforms often can not be properly identified because of the effect of salt on the separation pattern. To counteract these problems, it is usually recommended to desalt the sample prior to separation (Wu, 1995). On-line (Clarke *et al.*, 1997) (Clarke, Tomlinson et al., 1997) and off-line (Cifuentes, Moreno-Arribas et al., 1999) desalting have been tested for CIEF of erythropoietin glycoforms. It has been then concluded that for sample with high salt content, off-line desalting before introducing the sample into the capillary was preferred to an on-line desalting step.

2.1.9. One-Step CIEF

One-step CIEF was introduced as early as 1992 (Krull and Mazzeo, 1992) permitting to reduce run times from 30 min for a two-step CIEF to 5 min through reversing the polarity (cathode at the end of the capillary) and shortening the separation distance.

Since then, this approach has been exploited by Moorhouse *et al.* (Moorhouse, Eusebio et al., 1995) to further improve the separation of the rt-PA glycoforms in less than 10 min. A neutral, coated capillary of 50 μm I.D. was used with HPMC added to reduce EOF to a constant and reproducible value. A 50:50 mixture of two different ampholytes (pH=5-8 and pH=3-10) at a 3% level, 4M urea, 7.5% N,N,N'N'-tetramethylethylenediamine (TEMED) and 0.1% HPMC ensure the best resolution and the solubility of the glycoprotein throughout the focusing, resolving the sample into ten glycoforms. With the optimal conditions (ratio of the two ampholytes, sample concentration, the concentration of urea, HPMC and TEMED) inter-assay precision for migration time (<5%) and for normalized areas (<10%) was adequate. These authors have validated the above method with minor modifications (Moorhouse, Rickel et al., 1996) in a series of experiments examining accuracy, precision, specificity and ruggedness of the method.

Lee (Lee, 1997) developed a rapid (<5 min), simple and reproducible one-step CIEF to resolve isoforms and glycoforms of monoclonal antibodies and to determine their pI. The method permitted the monitoring of the lot-to-lot consistency and of the purification of MAbs. Fused-silica capillaries of 50 μm I.D. were used, with reverse polarity. Samples and standards were dissolved in ampholyte-HPMC mixtures, with or without addition of urea which was needed to solubilize MAbs. Analyses both with and without urea, using two different sets of reagents and capillaries and performed on three consecutive days, showed that the migration times of isoforms were highly reproducible. Linearity of pI calibration was also investigated: the use of urea resulted in correlation coefficients slightly lower than in absence of urea, probably due to the pI modification of protein standards under (partially) denaturing conditions. The microheterogeneity

fingerprints of MAbs can thus be determined and monitored in a simple, rapid and economical way.

More recently, our group developed a one-step CIEF method to resolve the glycoforms of the heterogeneous recombinant human immunodeficiency virus envelope glycoprotein (rgp 160) in 5 min (Tran, 2000). A typical glycoform pattern was obtained using an optimized mixture of ampholytes (narrow and wide pH range) and a combination of sucrose and 3-(cyclohexylamino)-1-propane-sulfonic acid which was shown to be the most efficient additive to avoid protein precipitation (see Figure 2). However, although the reproducibility of rgp160 separation depicted in Figure 2B, C and D was then confirmed, spikes appeared sometimes, indicating that the protein insolubility phenomenon was not completely resolved. The profiles obtained under optimal conditions for five standard markers with pI values ranging from 2.75 to 9.45 is also

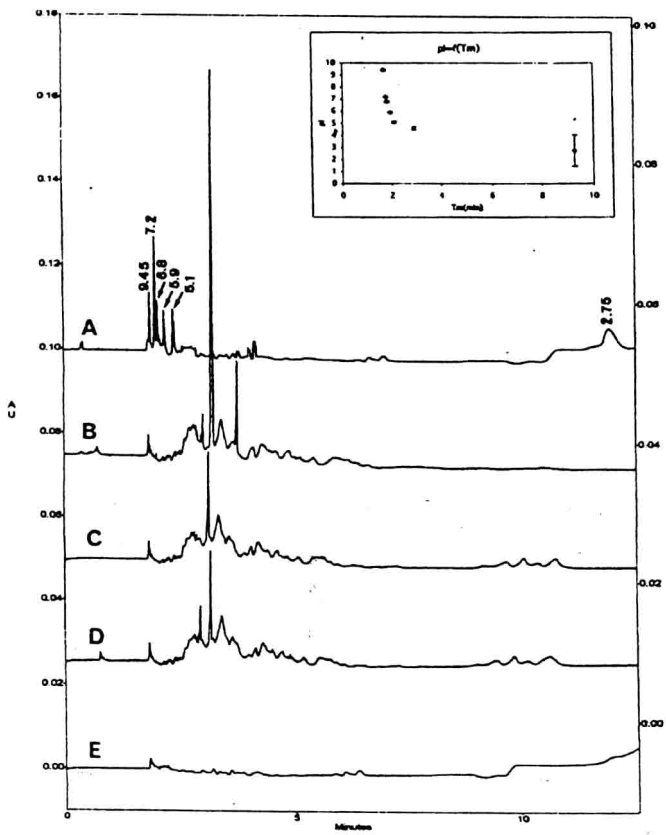

Figure 2. **(A)** One-step CIEF electrophoregram of protein standards. **(B-D)** Reproducibility of the separation of rgp 160 by the one-step CIEF. (E) Backgroung electrophoregram. **Inset:** Calibration curve (pI versus migration times) made from the mean of six analyses of the protein standards, bars indicate the corresponding SD. **Conditions**: PVA coated capillary 50µm×47cm; 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, 0.085M 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 pI standards markers; Anolyte: 100 mM H_3PO_4; Catyholyte: 20 mM NaOH; Focusing and mobilization at –20 kV.

shown in Figure 2. Although the calibration curve (isoelectric point *versus* migration time displayed in the inset of Figure 2) showed a non-linear relationship, an adequate linearity could be yielded over a narrow range of pH as previously discussed by Schwer (Schwer, 1995). However, this relation permitted to exhibit the acidic character of the different glycoforms of rgp 160 (pI from 4.00 to 4.95). Equivalent resolution was observed using PVA or PAA coated capillaries but PAA capillary showed longer migration time and a profile more extended, indicating that PVA capillary exhibited a higher residual EOF. It has been shown that not only the intra-day reproducibility but also the long term stability were lower in the case of the PAA capillary. The application of this method to a comparison of glycoform patterns of the rgp 160 of two sub-populations (or clades) of the virus HIV-1 suggested that one clade exhibited not only a lower microheterogeneity but had also more acidic glycoforms.

2.1.10. Two-Step CIEF

A two-step method with pressure mobilization was utilized for the routine analysis of rtPA (Thorne, Goetzinger et al., 1996) Using PAA coated capillary, 50 µm I.D., ampholine as carrier ampholyte and denaturing conditions produced by the addition of urea, rtPA was resolved into at least eight species, with pI values ranging from 6.4 to 9.2. The developed method is shown to be validatable: acceptable total protein recovery from the capillary (93%, n=3) and method reproducibility (RSD 2-3%, n=17 for the four major peaks) were demonstrated. Migration time precision was shown to be considerably improved if the glycoform peaks are bracketed with peptide pI markers, and their migration time calculated relative to the marker peaks.

Two-step CIEF has been used for monitoring recombinant human interferon-γ (IFN-γ) N-glycosylation during perfused-fluidized-bed and strirred-tank batch culture of CHO cells.(Kopp, Schluter et al., 1996). At least 11 differently sialylated glycoforms over a pI range of 3.4 to 6.4 have been resolved. Desialylation of the protein of a non specific neuraminidase resulted in a major shift in the molecular pI to a narrow peak grouping with pI values between 8.3 and 9.6. It has been shown that the degree of sialylation had a marked effect on the apparent pI of rIFN-γ. For quantitative analysis, the relative proportion of acidic rIFN-γ glycoforms increased after 210 h of culture, indicating an increase in N-glycan sialylation during establishment of the perfusion culture.

Two-step CIEF has been used for characterizing the purified recombinant antithrombin III (rATIII) produced in BHK cells by its isoelectric point (Reif and Freitag, 1995). Using a dextran-coated capillary 50µm I.D. and a mixture of four differents ampholytes (2% Ampholine pH 4-6, 0.5% Pharmalyte pH 3-10, 0.5% Pharmalyte pH 2.5-5, 0.5% Pharmalyte pH 4-6.5) in the presence of 0.01% HPMC, 0.1% TEMED and 0.001% triton X-100, hydrodynamic mobilisation allowed a pattern of six fractions focused in the pH range 4.7-5.3. It is important to note that desalting of the sample was crucial for the correctness of the determined pI.

In-process control in the production of ATIII and human clotting factor IX (FIX), two human plasma glycoproteins from the blood clotting cascade, has been investigated by CIEF (Buchacher, Schulz et al., 1998; Hunt, Hotaling et al., 1998) CIEF of the two glycoproteins turned out to require rather different measuring conditions: rATIII gave