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Journal of Chromatography B, 697 (1997) 123–133

JOURNAL OF CHROMATOGRAPHY B

# Combination of capillary electrophoresis and matrix-assisted laser desorption ionization mass spectrometry for glycosylation analysis of a human monoclonal anti-Rhesus(D) antibody

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**Abstract**

Characterization of a human anti-Rhesus(D) monoclonal antibody, developed for the treatment of Rh(D) haemolytic disease of the newborn, was performed. Capillary electrophoresis (CE) has been employed for peptide mapping of the IgG heavy chain and glycopeptide identification. The combination of the high resolution and low solvent consumption of CE and the ultrasensitive detection and precise identification properties of mass spectrometry led to a complete glycosylation analysis of the protein. Glycopeptides were easily isolated from a single injection in a 100 μm I.D. capillary of the preparative CE system and collected for molecular mass determination using matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). The off-line CE–MS characterization revealed the presence of different oligosaccharides linked to the unique $N^{297}$-S-T glycosylation site of the IgG heavy chain. The differences between calculated and experimental masses of the glycopeptides suggested the presence of a fucosylated biantennary structure containing one or two galactose units as major oligosaccharide, together with similar species bearing a bisecting N-acetylglucosamine. CE conditions were optimized to allow the MS identification of sialylated forms. © 1997 Elsevier Science B.V.

*Keywords:* Peptide mapping; Anti-Rhesus(D) monoclonal antibodies; Glycopeptides

## 1. Introduction

Foetomaternal alloimmunization of pregnant Rhesus (Rh) negative women is successfully prevented by routine administration of anti-Rh(D) antibodies reducing the occurrence of haemolytic disease of the Rh positive newborn. The diminishing availability, the viral safety and the increased demand explain the trend in the recent years to replace the polyclonal antibody by a cell culture-derived human monoclonal anti-D immunoglobulin [1,2]. The development of monoclonal antibodies for therapeutic use requires an extensive physico-biochemical and biological characterization of the purified protein for testing the steadiness of the manufacturing process and the consistency of the product. Among the different analytical methods for evaluating these physico-biochemical properties, peptide mapping is an available technique for sequence verification and identification of post-translational modifications such as glycosylation. The glycosylation of immunoglobulins has a strong influence on their biological

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*PII* S0378-4347(97)00305-8

functions. Moreover, it has been already described that the glycosylation pattern depends on the clone, the culture conditions and the downstream processes and that changes in glycosylation pattern may lead to dramatic dysfunctions in the immune response [3,4].

This primary sequence analysis using capillary electrophoresis (CE) represents an important application, already extensively reviewed [5,6]. CE can be performed in a two-dimensional separation system based on its use in combination with a complementary reversed-phase high-performance liquid chromatography (RP-HPLC) technique by exploiting a different mechanism for the separation of peptides. In our previous work, we described the peptide mapping of a monoclonal antibody by free solution capillary electrophoresis (FSCE) with increased resolution obtained at acidic pH and in the presence of hexane sulfonic acid (HSA) [7]. Recently, Rush et al. [8] described the peptide mapping and the glycopeptide microheterogeneity characterization of recombinant human erythropoietin using heptane sulfonic acid. The rationale for employing an ion pairing agent is to minimize the interactions between the capillary wall and the solute, to increase the resolution of tryptic peptides, and to separate the glycopeptides from the bulk of unmodified peptides [8]. Based on these observations, peptide mapping of the glycosylated heavy chain of the anti-Rh(D) antibody was performed by CE in the presence of HSA.

Combining the benefits of CE analysis with a mass spectrometric technique was the next step to achieve in order to characterize the glycosylation pattern of the anti-Rh(D) antibody. A first possibility may involve an on-line combination of electrospray mass spectrometry with CE, a solution that has already shown proven performances [9]. A second possibility involves matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS). The recent introduction of sources working with delayed extraction has considerably improved the accuracy of the technique [10] making it even more attractive for glycosylation analysis, although on-line CE coupling remains problematic. Indeed, a mass accuracy close to 10 ppm may be attained with a MALDI mass spectrometer within the mass range useful for peptide analysis. The amount of sample can be kept below 100 fmol [11], allowing at the same time to run post source decay (PSD) and collision induced dissociation (CID) analyses, thus leading to a very detailed characterization of glycosylated peptides. Therefore, we describe in this work the first study of antibody glycosylation by off-line CE–MALDI-MS, using fraction collection from a single injection of the protein tryptic digest.

## 2. Experimental

### 2.1. Materials

The monoclonal anti-Rh(D) antibody was secreted by a human lymphoblastoïd cell F5 [12] in a hollow-fiber cartridge in presence of a serum free medium [13]. After the purification process, the immunoglobulin was stored in a 150 m*M* alanine, 100 m*M* NaCl buffer, pH 6.8, at 4°C.

1-Hexane sulfonic acid (HSA), Tris-(hydroxymethyl)aminomethane hydrochloride, ammonium acetate and α-cyano-4-hydroxycinnamic acid were purchased from Sigma (St. Quentin Fallavier, France). Acetonitrile and trifluoroacetic acid (TFA) were from J.T. Baker (Noisy le Sec, France). Urea, trypsin, anhydrous disodium tetraborate were obtained from Merck (Darmstadt, Germany). Dithiothreitol (DTT)–N,N′-methylenebisacrylamide solution (37.5:1), N,N,N′,N′-tetraethylenediamine (TEMED) ammonium persulfate and sodium dodecyl sulfate (SDS) were purchased from Bio-Rad (Ivry sur Seine, France). All reagents were HPLC-grade or analytical quality. The buffers were filtered through a 0.22 μm Millex membrane and degassed before use.

### 2.2. Preparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Preparative SDS–PAGE was run according to the discontinuous buffer system of Laemmli [14] using the Model 491 Prep Cell (Bio-Rad). The upper and lower electrophoresis chambers of the apparatus were filled with a 25 m*M* Tris–HCl, 192 m*M* glycine, 0.1% SDS buffer, pH 8.3. The lower chamber buffer circulated at 100 ml/min at 15°C. A sample was prepared by mixing 20 mg (5 ml) of the

purified antibody with 4 ml of sample buffer (50 m*M* Tris–HCl, 20% glycerol, 10% SDS, 5% β-mercaptoethanol and 0.01% bromophenol blue) and heating at 95°C for 5 min. A 9 cm high, 8% acrylamide separating gel and a 2 cm high, 4% acrylamide stacking gel were polymerized in the 37 mm diameter Model 491 Prep Cell casting tube assembly. The electrophoresis was performed at a constant current of 13 mA for the first 5 h and at 20 mA constant current for the remainder of the electrophoretic separation. After elution of the dye front (16 h), fractions (5.6 ml) were collected at 0.4 ml/min in a 25 m*M* Tris–HCl, 192 m*M* glycine buffer, 20% methanol, pH 8.3. Eluted proteins were detected on line at 280 nm. Fractions collected from the preparative electrophoresis were characterized by SDS–PAGE. Purified heavy chains were dialysed and lyophilized before storage at 4°C.

### 2.3. Electrophoresis and staining methods

When proteins are deglycosylated, a mobility shift can be observed by SDS–PAGE and the magnitude of apparent molecular mass decrease gives a rough estimation of the amount of N-linked chains in a glycoprotein. SDS–PAGE was conducted according to the method described by Laemmli [14].

#### 2.3.1. Periodic acid Schiff (PAS) staining

This staining method was developed according to Carlsson [15]. After migration, the gel was incubated in a methanol–acetic acid–water (35:10:55) bath for 1 h. Then, the gel was incubated in a periodate solution [0.7 g periodic acid in 100 ml of 5% (v/v) acetic acid]. This step was followed by a bath of sodium metabisulfite solution [0.2 g in 100 ml of 5% (v/v) acetic acid] for 10 min. The staining was carried out in a bath of Schiff's reagent (pararosaline chloride 1%, sodium bisulfite 4%, in 250 m*M* HCl, Sigma) until the appearance of red bands.

### 2.4. Enzymatic deglycosylation

N-Glycanase (peptide-N4-[N-acetyl-b-glucosaminyl] asparagine amidase), also called peptide N-glycosidase (PNGase), purified from cultures of *Flavobacterium meningosepticum*, cleaves all classes of N-linked carbohydrate chains at the peptide–carbohydrate linkage, leaving aspartic acid at the glycosylation site. Recombinant PNGase F (200 U/ml; 25 000 U/mg, Boehringer Mannheim, Meylan, France) was used for all enzymatic deglycosylations. The reaction was conducted in a 50 m*M* sodium phosphate buffer pH 7.2 [16] with native glycoproteins or heavy and light chains previously separated by preparative electrophoresis. Purified tryptic peptides were diluted with 250m*M* bisTris–propane buffer, pH 8.3. IgG heavy chains, or tryptic peptides were deglycosylated after incubation at 37°C for 18 h with PNGase F (50 mU/μg). Under these conditions all N-linked oligosaccharides were removed as indicated by MALDI-MS and SDS–PAGE analysis.

### 2.5. Proteolytic cleavage

Anti-Rhesus(D) antibody heavy chain (1 mg) was denatured in 8 *M* urea, 0.4 *M* ammonium hydrogenocarbonate solution, reduced by dithiothreitol (20 mol DTT/mol cysteine) at 50°C for 15 min and alkylated by iodoacetamide (2 mol/mol DTT, 15 min in the dark at room temperature). After a four-fold dilution with water, hydrolysis with trypsin (1:10, w/w) followed for 18 h at 37°C [17].

### 2.6. Peptide mapping by CE

Analytical CE runs were performed with an Applied Biosystems instrument (Model 270A), using a 72 cm×75 μm I.D. capillary. We used a noncoated, fused-silica capillary with built-in Z-shaped flow cell to enhance optical detection. Before each injection, the capillary was rinsed with 0.1 *M* NaOH, and flushed for 5 min with a 30 m*M* $Na_2HPO_4$–$H_3PO_4$ running buffer, pH 2.5, containing 50 m*M* HSA. Electrophoresis was carried out at 20 kV and 30°C and peptides were monitored at 200 nm.

### 2.7. Peptide mapping by RP-HPLC

RP-HPLC was performed on a Waters HPLC system (pump 510 and detector 484) using a $C_{18}$ column (Inertsil ODS2, 5 μm particles, 150 Å pore size, 100×3.2 mm, Interchim). The column was

equilibrated with 0.115% TFA (buffer A). Buffer B was 0.1% TFA in acetonitrile. Elution was performed at 30°C under the following conditions: flow-rate of 0.5 ml/min, 0% B for 15 min after injection followed by linear gradients from 0 to 12% B in 12 min, then 12 to 42% B in 120 min and 42 to 80% B in 10 min. Monitoring was at 214 nm.

### 2.8. Preparative CE

Preparative CE was performed with a Beckman instrument (PACE system 5500), using a 57 cm×100 μm I.D. capillary and the buffer used for analytical runs or a 25 m*M* borate buffer, pH 9.0. Purified glycopeptides were concentrated five-fold, to 100 pmol/μl, and injected in the capillary during 15 s using the "low pressure" mode. Electrophoresis was carried out at 10 kV for 60 min and peptides were monitored at 200 nm. After selecting a desired migration time window, the system stopped the voltage and changed the outlet buffer vial to a collection vial. Fraction collection was achieved in water (2.5 μl), over 30 s using the low pressure mode. This migration time window was determined considering the time for glycopeptides detection (between 56 and 57.5 min after injection), the capillary length to the detector (50 cm) and the rate corresponding to the "low pressure mode" (50 cm/222 s). After the desired peak was eluted, the system changed the vials and reapplied the voltage.

### 2.9. MALDI-MS

Collected fractions were diluted ten-fold in 0.1% TFA leading to a final concentration of 1 pmol/μl. Samples were prepared by mixing in an Eppendorf microtube 1 μl of matrix (50 m*M* solution of α-cyano-4-hydroxycinnamic acid in acetonitrile–ethanol 1:1, v/v) with 1 μl of digest. A 1 μl volume was then loaded onto a stainless-steel multi-sample holder and dried at room temperature. A VG Analytical Tofspec mass spectrometer equipped with a 337 nm laser was used for sample analysis, with a 25 kV acceleration voltage. Between 50 and 100 shots were accumulated for each spectrum acquisition in the positive ion mode. External standards for mass calibration were bradykinin and bovine insulin.

## 3. Results and discussion

### 3.1. Purification of the heavy chains

Preparative SDS–PAGE was tested as an alternative method to liquid chromatography for purification of the heavy chains of the reduced anti-Rh(D) antibody. Various experimental conditions such as, the amount of loaded protein, gel length, gel porosity and the electric potential have been explored to optimize the resolution. Two peaks were clearly separated (Fig. 1, peaks 1 and 2) with a purification yield of 60%. A 12 mg quantity of purified heavy chains was recovered (peak 2 collected over 210 min) from 20 mg of loaded IgG. Aliquots of the corresponding eluted fractions were analyzed by SDS–PAGE. Fig. 1 (inset) shows that peaks 1 and 2 corresponded to the purified heavy and light chains at 50 000 and 25 000, respectively (lanes b and c) obtained from the reduced $IgG_1$ (lane a). The absence of contaminating bands on the gel reflects the high purity of the polypeptides obtained by preparative SDS–PAGE, further confirmed by MALDI-MS (data not shown).

### 3.2. Endoglycosidase sensitivity of the IGg heavy and light chains

Fig. 2 illustrates the SDS–PAGE analysis of the reduced forms of the human anti-Rh(D) antibody. Coomassie blue staining revealed the presence of the heavy and light chains at 50 000 and 25 000, respectively (lane 1). After specific polysaccharide staining by PAS, only the heavy chain was revealed (lane 3). Specific glycosidases have been used to determine the type of glycosylation. Following N-glycanase treatment, the 50 000 heavy chain exhibits a decrease in mass, while no mobility change occurs for the 25 000 light chain (lane 2). Furthermore, after this enzymatic cleavage the sensitivity of the 50 000 protein to PAS staining was lost (lane 4). These results indicated the presence of oligosaccharides N-linked to the heavy chain of the anti-Rh(D) antibody.

### 3.3. Peptide mapping by CE and RP-HPLC

FSCE was used as a high-resolution technique for peptide mapping of purified heavy chain of the

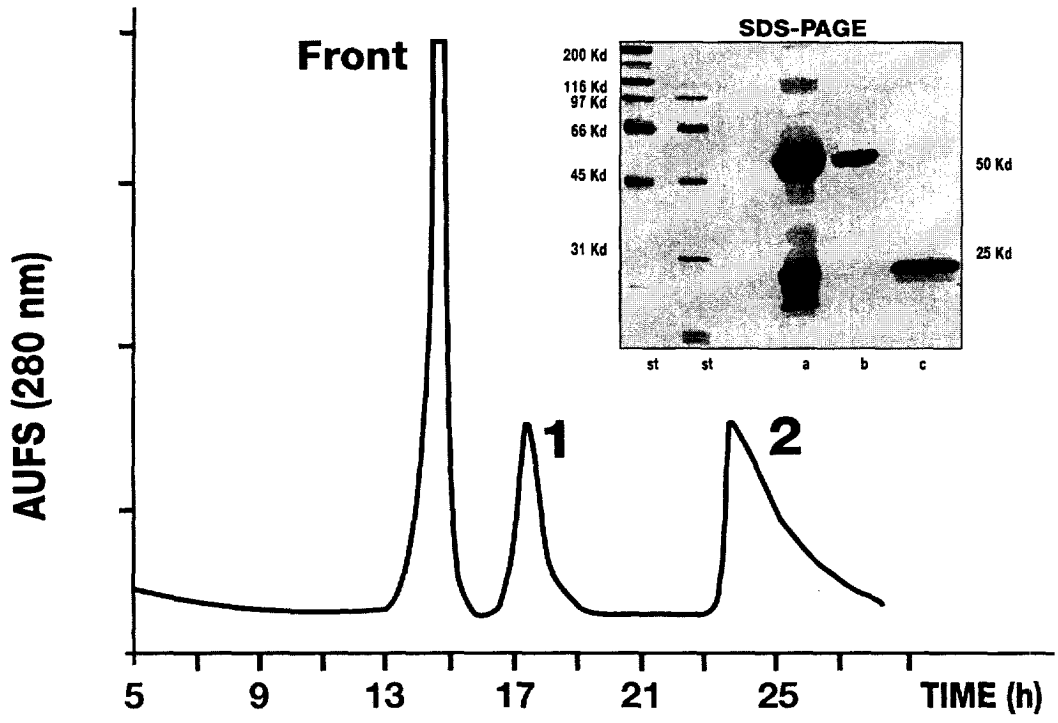


Fig. 1. Purification of the anti-Rh(D) antibody heavy chain by preparative SDS–PAGE. Proteins were eluted at 0.4 ml/min with the Tris–HCl, glycine, buffer, pH 8.3, containing 20% methanol. Purified light chains (peak 1) and heavy chains (peak 2) were detected at 280 nm and collected for SDS–PAGE analysis (inset). Lanes (st), (a), (b) and (c) represent molecular mass markers, the reduced $IgG_1$ (100 μg loaded), the 50 000 heavy chains and 25 000 light chains (15 μg loaded), respectively.

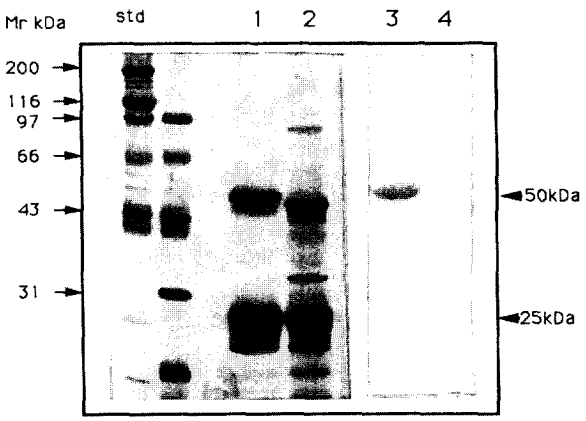


Fig. 2. Deglycosylation analysis of the anti-Rh(D) IgG by SDS–PAGE. Lanes (st), (1) and (2) represent, on the Coomassie blue stained gel, the molecular mass markers, the reduced $IgG_1$ and its deglycosylated form, respectively (15 μg loaded). The glycosylated (lane 3) and deglycosylated (lane 4) forms of the reduced antibody have been also characterized by SDS–PAGE and PAS staining (30 μg loaded).

anti-Rh(D) antibody. In the absence of surfactant in the running buffer, the tryptic peptides were poorly resolved, while in the presence of an ion-pairing agent, such as alkyl sulfonate, the resolution was greatly improved by decreasing the mobility of the peptides and reducing the solute–wall interactions. Fig. 3 shows a typical peptide map of the antibody heavy chain obtained in the presence of 50 m*M* HSA in the running buffer. The FSCE sensitivity has been assessed using a high-sensitivity optical Z-cell, by identification of the different peaks of the map ($S/N=3$) after injection of 6 fmol of the digested polypeptide (Fig. 3). The reproducibility of the maps obtained from different antibody preparations is important for the batch-to-batch consistency of the molecule and a precise identification of the glycopeptides.

Fig. 4 shows the correlation between the peptide maps obtained by RP-HPLC and FSCE. The major RP-HPLC peaks are numbered according to their elution time and each corresponding FSCE peak is

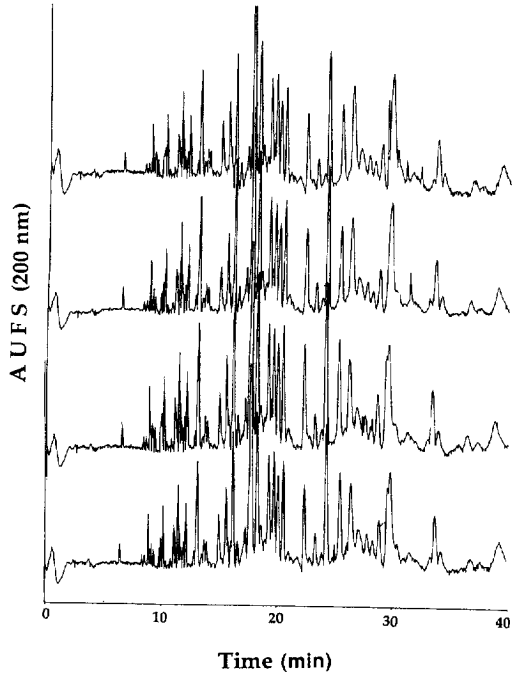


Fig. 3. Assessment of reproducibility of peptide mapping of the antibody heavy chain by CE. Spectra represent four separate sample preparations. Conditions: field, 20 kV; running buffer, 30 m*M* $Na_2HPO_4$–$H_3PO_4$, pH 2.5, containing 50 m*M* HSA. Detection was at 200 nm using a high-sensitivity optical cell.

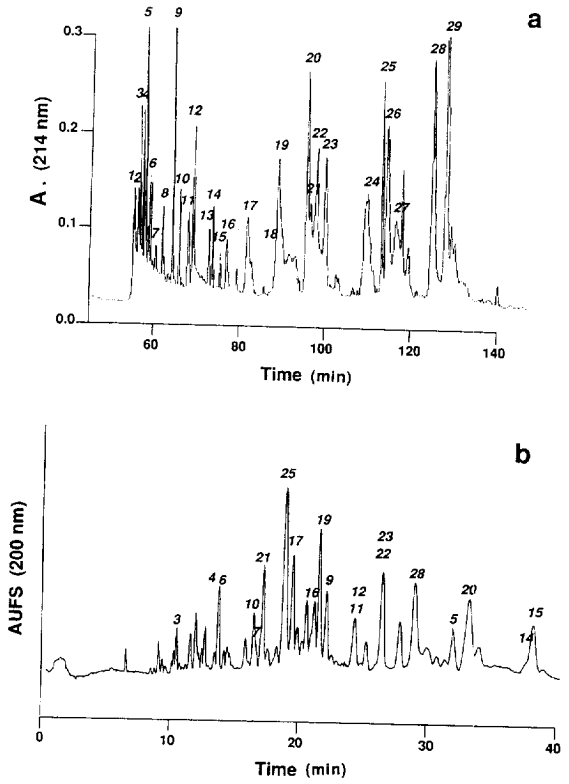


Fig. 4. (a) Peptide mapping by RP-HPLC. Chromatographic conditions: column, Inertsil ODS2, 5 μm particles, 150 Å pore size (100×3.2 mm). Buffer A, 0.115% TFA in water. Buffer B, 0.1% TFA in acetonitrile. Flow-rate: 0.5 ml/min. Gradients: 0 to 12% B in 12 min, 12 to 42% B in 120 min and 42 to 80% B during 10 min. Detector: 214 nm wavelength. (b) Correlation with the map obtained by CE (same conditions as in Fig. 3): the major peptides obtained after RP-HPLC, numbered 1 to 29, were identified on the electropherogram.

identified by the same number. Considering the great number of peptides, a shallow acetonitrile gradient (slope 0.25%/min) has been used to obtain a good resolution. Under these conditions, separation took 160 min. The comparison of the chromatogram (Fig. 4a) and the electropherogram (Fig. 4b) indicated that almost all the peptides are separated by CE; only peptides 4 and 6, 11 and 12 and 22 and 23 overlapped. Fig. 4 indicates that there is no correlation between FSCE and RP-HPLC elution orders. Furthermore, peptides that are poorly separated by RP-HPLC, such as peptides 3, 4 and 5 or 20, 21 and 22 are well separated by CE. The analysis time is 4-times faster for FSCE. These results confirm that FSCE is a complementary technique to RP-HPLC, based on a different separation mechanism, for structural characterization of a molecule which gives a sensitive, quantitative, well resolved and reproducible map in a short run time.

### *3.4. Identification of the glycopeptide*

After trypsin digestion of the IgG heavy chain, the different peptides obtained have been separated by RP-HPLC and glycopeptide-containing peaks were identified in each eluted fraction after PNGase addition by observing a retention shift due to deglycosylation. Fig. 5 shows that, following N-glycanase treatment, peak 5 exhibits an increase in the retention time (peak 5d) while no shift was observed for the other eluted peaks (peaks 3 and 3d). The increased hydrophobicity is consistent with the loss of the oligosaccharides N-linked to peptide 5. Fur-

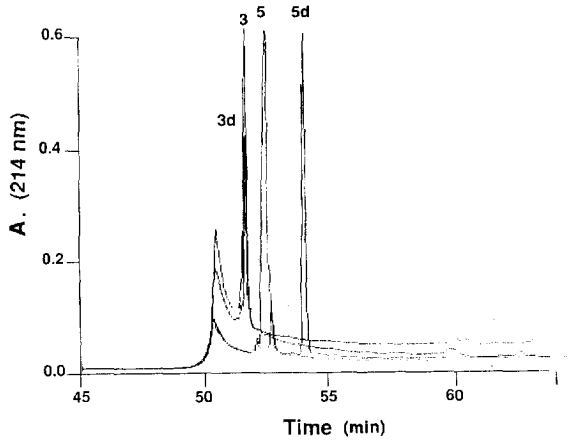


Fig. 5. Glycopeptide identification by RP-HPLC. Purified tryptic peptides were diluted with 250 m*M* bisTris–propane buffer, pH 8.3, deglycosylated by PNGase F (50 mU/μg) and characterized by RP-HPLC (same conditions as for Fig. 4). Chromatograms of deglycosylated peptides 3 and 5 (peaks 3d and 5d, respectively) and of the corresponding unmodified forms (peaks 3 and 5) are overlayed.

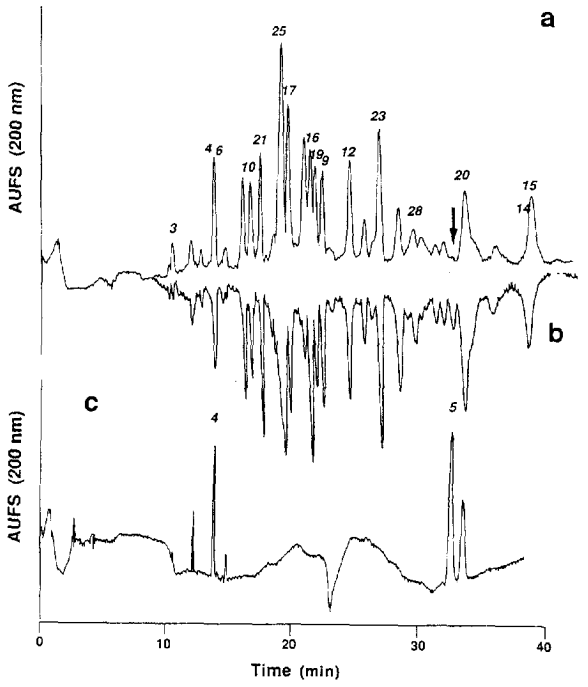


Fig. 6. Glycopeptide identification by CE. Overlay of the electropherograms corresponding to the peptide maps obtained with the deglycosylated (a) and the glycosylated (b) forms of the antibody heavy chain (same conditions as for Fig. 3). (c) CE analysis at 200 nm of the purified glycopeptides 5 (peptide 4 was used as an internal standard).

thermore, purified antibody heavy chains have been deglycosylated and compared to the untreated forms. Fig. 6 shows the different maps obtained after trypsin digestion and CE characterization of the glycosylated (Fig. 6a) and deglycosylated forms of the protein (Fig. 6b). This comparative analysis revealed that deglycosylation by the N-glycanase is correlated with the disappearance of peak 5. On the basis of these data and according to the amino acid sequence of the IgG heavy chain, peptide 5 corresponds to the tryptic glycopeptide [$E^{293}$-$R^{301}$] containing the unique N-glycosylation site ($N^{297}$-S-T) of the molecule. MS characterization was performed to identify the various oligosaccharides that could be linked to this peptide.

### *3.5. MALDI-MS analysis of the glycopeptides isolated by CE*

Fig. 7a (inset) represents the electropherogram obtained after preparative CE of the glycopeptide 5, performed with the buffer already used for peptide mapping. A single run allows the collection of a 10 pmol/μl sample of glycopeptides using a 100 μm I.D. capillary. MALDI-MS characterization of the CE fraction has been performed in the reflectron mode for a precise molecular mass determination. The off-line CE–MS analysis revealed the presence of four peaks (Fig. 7a), indicating that several oligosaccharides were linked to the glycosylation site of the IgG heavy chain. Differences between the experimental mass of each glycopeptide and the theoretical mass (1189.2 for a protonated molecule) of the unglycosylated tryptic peptide [$E^{293}$-$R^{301}$] led to the identification of different oligosaccharides linked to $N^{297}$. Fraction 5 reveals the occurrence of four neutral fucosylated biantennary oligosaccharides. The minor glycopeptide, at 3162.4 (Fig. 7a), is modified by a glycan at 1973.2 corresponding to a biantennary form bearing a bisecting N-acetylglucosamine (GlcNac) (theoretical mass: 1972.8). The molecular mass of 1811.8, deduced from the major species at 3001.0, corresponds to the

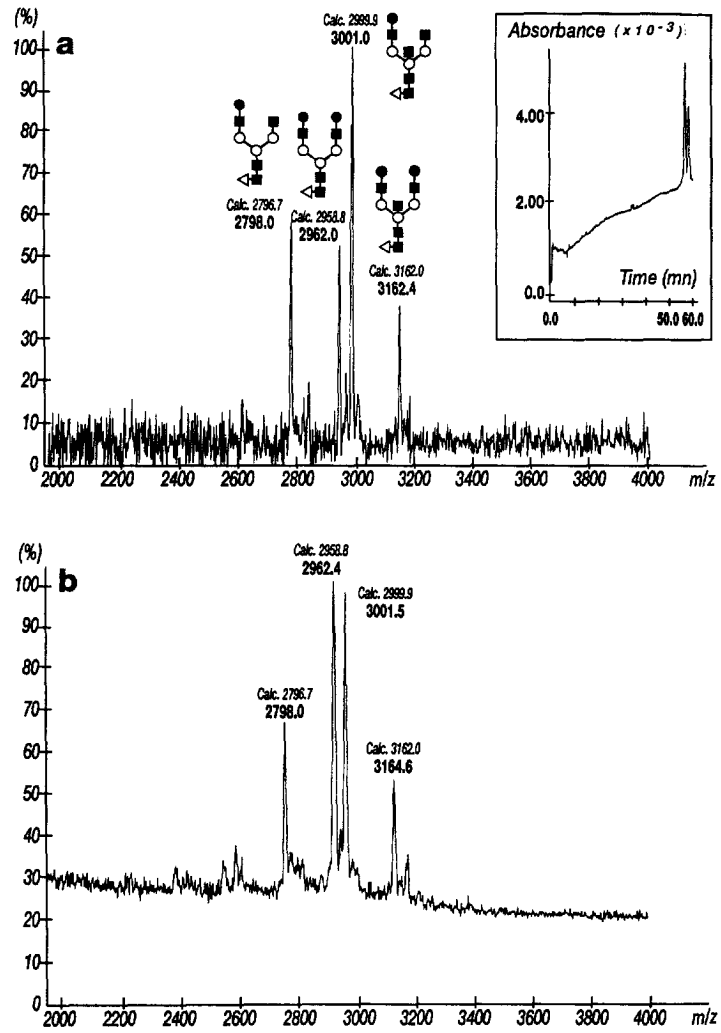


Fig. 7. Off-line CE–MS analysis of the glycopeptides. Preparative CE (a, inset) was performed using a 100 mm I.D. capillary and the buffer used for analytical runs. Electrophoresis was carried out at 10 kV during 60 min. Fraction collection was achieved in water using the low pressure mode. (a) MALDI-MS analysis of glycopeptides obtained by preparative CE. The time-of-flight mass spectrometer was operated in the reflectron mode at 25 kV acceleration and 29 kV reflectron voltage. Sample was loaded with α-cyano-4-hydroxycinnamic acid as a matrix. Monosaccharides symbols are: (△) fucose, (■) N-acetylglucosamine, (○) mannose, (●) galactose. (b) MALDI–MS characterization of the same glycopeptides purified by RP-HPLC.

same glycan missing a terminal galactose (theoretical mass: 1810.7). Furthermore, glycopeptides at 2962.0 and 2798.0 (Fig. 7a) both miss the bisecting GlcNac and differ by the terminal galactose (162.1). Off-line HPLC–MS analysis of fraction 5 allowed identification of the same four glycopeptides (Fig. 7b). However, the major species are modified by biantennary fucosylated glycans missing the bisecting GlcNac (measured mass: 2962.4).

Since the reflectron mode could induce oligosaccharide fragmentation, MALDI-MS characterization has been performed in the linear mode for identification of the unstable sialylated forms (Fig. 8). The mass spectrum obtained after CE–MS analy-

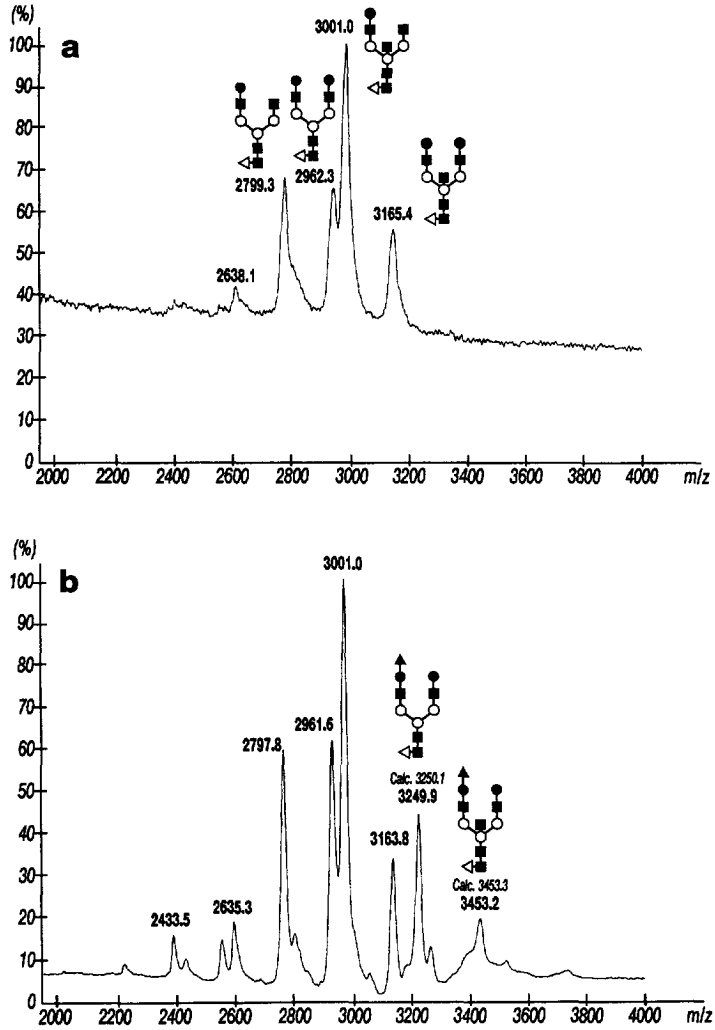


Fig. 8. Off-line CE–MS analysis of the glycopeptides. The time-of-flight mass spectrometer was operated in the linear mode at 25 kV acceleration voltage. MALDI-MS analysis was performed with glycopeptides obtained by preparative CE (a) or RP-HPLC (b). Monosaccharide symbols are as for Fig. 7.

sis (Fig. 8a) was similar to the preceding ones suggesting the absence of sialylated glycans. By contrast, HPLC–MS characterization revealed the presence of additional mass peaks at 3249.9 and 3453.2 (Fig. 8b), corresponding to different sialylated glycopeptides. HPLC–MS results thus confirm that MALDI-MS analysis performed in the reflectron mode induces glycan desialylation. A comparison of mass spectra indicates that the higher abundance of the peak at 2962.4 observed in Fig. 7b is consistent with desialylation (loss of 291.2) of the corresponding monosialylated form at 3249.9 (Fig. 8b).

The absence of sialylated glycopeptides observed in Fig. 8a could be explained by the use of an acidic buffer (pH 2.5) for CE analysis. Fig. 9b represents the MALDI-MS characterization of glycopeptides isolated by preparative CE (Fig. 9a) run with borate buffer (pH 9.0). Fig. 9b shows that among the four neutral forms already identified by MALDI-MS (Fig.

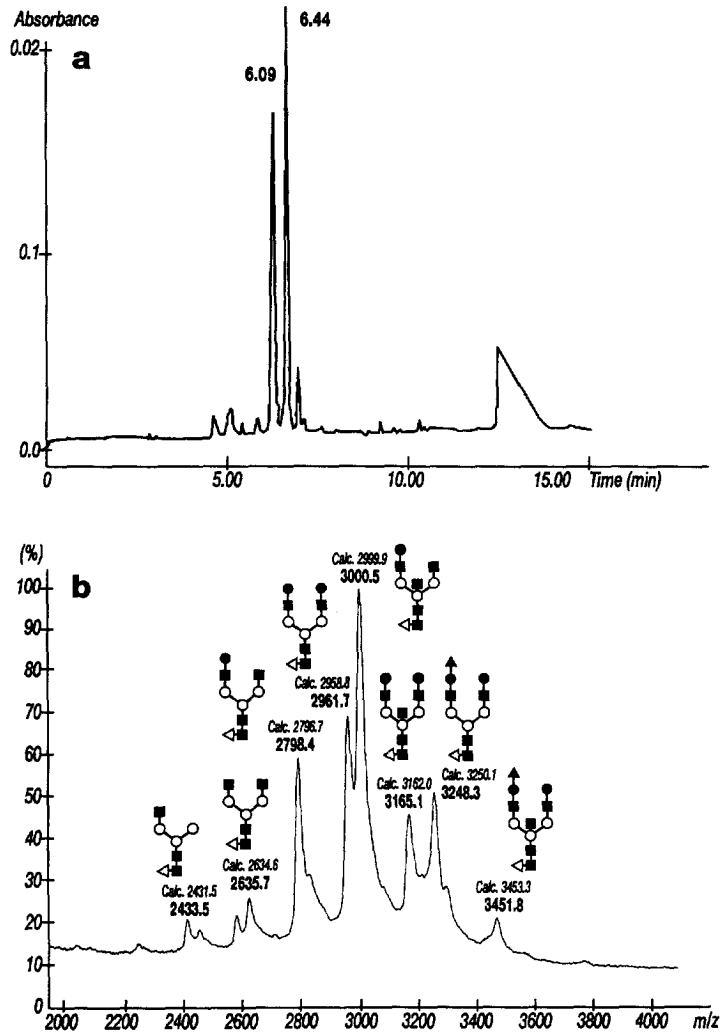


Fig. 9. Off-line CE–MS analysis of the glycopeptides isolated by RP-HPLC. (a) Preparative CE was performed using a 100 mm I.D. capillary and a 25 m*M* borate buffer (pH 9.0). Electrophoresis was carried out at 10 kV during 38 min. Fraction collection was achieved in water using the low pressure mode during 98 s. (b) MALDI-MS analysis (linear mode) of glycopeptides obtained by preparative CE. Monosaccharide symbols are as for Fig. 7 and (▲) sialic acids.

7), the major species is a the biantennary fucosylated oligosaccharide bearing a bisecting GlcNac and missing a galactose. Two fucosylated biantennary compounds account for monosialylated structures, indicating that this basic CE buffer avoids glycan desialylation. The major monosialylated glycopeptide (3248.3) is modified by fucosylated biantennary oligosaccharides, with an added mass of 2059.1 (theoretical mass: 2060.9).

## 4. Conclusions

CE performed in the presence of an ion-pairing agent such as HSA affords high resolution of peptide maps, thus confirming the results obtained by Rush et al. [8]. The low mobility of glycopeptides observed in CE provides a basis to discriminate tryptic glycopeptides from unmodified ones by the association of RP-HPLC and CE analysis. The combination

of fraction collection from a single CE injection, followed by MALDI-MS analysis, allows a rapid identification of glycan species with high sensitivity. Furthermore, progress is now expected to arise from MALDI-MS combinations working in a continuous flow-mode [18], with the goal of setting up an on-line CE–MALDI-MS coupling.

## Acknowledgments

The authors gratefully acknowledge Dr F. Dhainaut and Mr J. Lirochon for the preparation of the monoclonal anti-D antibody.

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