Table 1
Results of the determination of some limonoid glucosides in seeds of Nartjie (*Citrus nobilis*) and Lemon (*Citrus limon*)

17-β-D-Glucopyranoside of	Concentration[a] (mg per g of dried seed)	
	Nartjie	Lemon
Limonin	0.523 ± 0.006	0.19 ± 0.02
Nomilin	0.45 ± 0.03	0.440 ± 0.005

[a] Average values of 3 injections.

Citrus nobilis containing known levels of these compounds (see Table 1). The recovery of the limonoid glucosides by the MECC method described gave recoveries of 94.1% for limonin glucoside and 93.5% for nomilin glucoside. Precision tests for limonin glucoside and nomilin glucoside were carried out on the Beckman P/ACE 2000 instrument. The coefficients of variation of the migration time for limonin glucoside and nomilin glucoside (9 injections) were 0.92% and 1.35%, respectively. The coefficient of variation for the normalized peak areas for limonin glucoside and nomilin glucoside were 1.92% and 7.98%, respectively. These results indicate that MECC is sufficiently sensitive for the reproducible determination of limonoid glucosides in citrus seeds.

The study has shown that MECC can be applied successfully to separate limonoid glucosides. The analysis of some of the major limonoid glucosides present in citrus seeds demonstrates the applicability of the proposed method. Further, the technique offers high separation efficiencies, rapid analyses, low running costs and is aqueous rather than organic solvent based. All these are advantages over traditional chromatographic procedures.

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Analysis of the glycoforms of human recombinant factor VIIa by capillary electrophoresis and high-performance liquid chromatography

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Abstract

The carbohydrate-dependent microheterogeneity of recombinant coagulation factor VIIa (rFVIIa) has been characterized by capillary electrophoresis (CE) of the native protein and by high-performance liquid chromatography (HPLC) of tryptic peptides and of oligosaccharides released by hydrazinolysis.

The development of the CE analysis is reported here. We have found that application of 1,4-diaminobutane (putrescine) as additive to the CE separation buffer is essential for the separation of the various glycoforms. Under optimum conditions rFVIIa migrates as a cluster of six peaks or more. By CE of neuraminidase-treated rFVIIa a faster-moving double peak is observed. This indicates that the separation obtained is primarily based upon the different content of N-acetyl-neuraminic acid of the oligosaccharide structures in rFVIIa.

By reversed-phase HPLC of tryptic digested neuraminidase treated rFVIIa the glycopeptides containing the heavy chain N-glycosylated site elute as two peaks compared to the four peaks corresponding to glycopeptides with 0 to 3 N-acetyl-neuraminic acids seen for untreated rFVIIa.

In high-pH anion-exchange HPLC of the oligosaccharides released from native rFVIIa by hydrazinolysis the major peaks elute as oligosaccharides with 1 or 2 N-acetyl-neuraminic acids. Oligosaccharides released from neuraminidase treated rFVIIa elute earlier compared to oligosaccharides from native rFVIIa, but separated into several peaks, indicating heterogeneity for the oligosaccharide structures without N-acetyl-neuraminic acid.

1. Introduction

Factor VII is a single-chain, vitamin K-dependent plasma glycoprotein that participates in the extrinsic pathway of blood coagulation. By the activation of factor VII to factor VIIa the peptide chain is cleaved between Arg152 and Ile153, resulting in a two-chain molecule consisting of a light chain (AA 1–152) and a heavy chain (AA 153–406) linked through one disulphide bond [1]. Human recombinant blood coagulation factor VII has been obtained from a mammalian expression system and has in its activated form (rFVIIa) been purified and characterized [1]. rFVIIa is post-translationally modified in several ways, including γ-carboxylation, O-glycosylation at Ser52 and Ser60 and N-glycosylation at Asn145 and Asn322 [1,2]. At Ser52 three different structures, $(Xyl)_{0-2}$-Glc-, and at Ser60 a single Fuc have been found [2].

Each N-glycosylated site in eukaryotic glycoproteins is associated with several different oligo-

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saccharide structures, also referred to as N-glycosylation microheterogeneity [3]. A glycoprotein with multiple N-glycosylated sites, therefore, consists of a population of glycoforms [3,4]. Since separation of such a glycoprotein into its individual glycoforms is not readily attainable [4], characterization of the glycosylation of glycoproteins has been performed after initial enzymatic or chemical cleavage [5–12]. A common approach for characterization of glycoprotein glycosylation has been proteolytic digestion followed by glycopeptide analysis using reversed-phase high-performance liquid chromatography (RP-HPLC) with detection by UV absorbance [5] or mass spectrometry [6]. Another approach has been to release the oligosaccharides by enzymatic [7,8] or chemical cleavage [8,9] and to analyze the oligosaccharides by high-pH anion-exchange HPLC (HPAEC) with pulsed amperometric detection (PAD) [7,8], by mass spectrometry [10], or by NMR [11,12].

HPLC separation of native glycoproteins with multiple N-glycosylated sites into fractions differing in the glycosylation is not readily attainable [3] and has to our knowledge only been obtained in a few cases. Frenz et al. [13] used anion-exchange HPLC to separate recombinant human deoxyribonuclease I glycoforms according to extent and positions of phosphorylation. In addition, some separation according to sialylation was obtained.

During the past three to four years several papers have been published concerning the application of capillary electrophoresis (CE) for the analysis of the carbohydrate-mediated heterogeneity of various glycoproteins. Some of the published work has been reviewed [14]. Relatively few glycoproteins have been addressed in the published articles, but separation of the glycoforms has been achieved using various analytical conditions. Capillary zone electrophoresis (CZE), as well as capillary isoelectric focusing and micellar electrokinetic capillary chromatography have been applied [15–23]. CZE using various buffers of both basic and acidic pH values seems to be the most preferred technique, probably due to its simplicity. Recent studies [24–26] have indicated that CZE using α,ω-diaminoalkanes like 1,4-diaminobutane (putrescine) as buffer additive may be general applicable for the separation of glycoforms.

In several papers it has been demonstrated that addition of 1,3-, 1,4- or 1,5-diaminoalkanes to the running buffer improves the resolution of proteins and polypeptides [27–31]. The beneficial effect of these polyethylenediamines is obviously due to a binding to the fused-silica wall. Thereby the electroendosmotic flow (EOF), as well as the interaction between the solutes and the capillary wall, is reduced. Whereas low levels (maximum 5 m*M*) of putrescine or cadaverine (1,5-diaminopentane) have been used in some cases [27–29], Bullock and Yuan [30] found that about ten times higher concentrations of 1,3-diaminopropane (30–60 m*M*) were best for the separation of a series of six basic proteins. These conditions have also proven useful in a subsequent paper on recombinant human protein interleukin-4 [31].

It is apparent from the above-cited literature that analysis of the carbohydrate-dependent microheterogeneity of glycoproteins is not readily attainable by use of HPLC while several good results have been obtained in recent years by use of various CE techniques. CZE seems to be the most preferred technique, probably due to its simplicity. While HPLC is of limited success with regard to separation of the glycoforms of native glycoproteins, various HPLC separation modes are essential for the characterization and purification of glycopeptides and oligosaccharides.

In the study presented here, we have investigated the separation of glycoforms of native rFVIIa by CE using a phosphate buffer with putrescine as buffer additive. The effect of other buffer parameters is also investigated. Native and neuraminidase treated rFVIIa are characterized by CE, RP-HPLC of tryptic glycopeptides, and HPAEC of released oligosaccharides.

2. Experimental

2.1. Materials

rFVIIa was expressed and purified at Novo Nordisk A/S (1). Trypsin (bovine, T-1005),

Neu5Ac (A-2751) and putrescine dihydrochloride (1,4-diaminobutane, 2HCl) were from Sigma (St. Louis, MO, USA). Neuraminidase was from Boehringer Mannheim (Mannheim, Germany) for rFVIIa and from Oxford GlycoSystems (X-5011, Oxford, UK) for the oligosaccharides. The reagent kit (I-4011) used for the hydrazinolysis and the oligosaccharide standards (C-335300, C-224300 and C-124300) were from Oxford GlycoSystems (Oxford, UK). All other chemicals were of analytical grade.

2.2. Neuraminidase treatment of r-factor VIIa and purified oligosaccharides

Removal of Neu5Ac was done by incubation of rFVIIa or purified oligosaccharide with neuraminidase in 50 m*M* sodium acetate, 5 m*M* calcium chloride, pH 4.8, for 3 h at room temperature. The enzyme–substrate ratio was 1:5 for rFVIIa and 1 U per approximately 100 pmol for the oligosaccharides.

2.3. Preparation and separation of r-factor VIIa tryptic peptides

rFVIIa samples (1 mg/ml) were digested with trypsin (enzyme/substrate ratio 1:100) in 50 m*M* Tris buffer, 5 m*M* $CaCl_2$, pH 7.5, for 20 h at 37°C. Digestion was terminated by addition of glacial acetic acid, adjusting the pH to approximately 3.5. The digestion product (25 μl) was analyzed using RP-HPLC: HPLC equipment, Waters; column, Nucleosil C_{18} column (250 × 4 mm, particle size 7 μm, pore diameter 12 nm, Macherey-Nagel 720042); column temperature, 30°C; detection, 215 nm; flow-rate, 1.5 ml/min; eluent A, 0.065% (by volume) trifluoroacetic acid in water; eluent B, 0.05% (by volume) trifluoroacetic acid in 90% acetonitrile in water. Gradient for heavy chain glycopeptides, 6–41% eluent B for 49 min; gradient for light chain glycopeptides, 0–16% eluent B for 40 min.

The elution positions of the glycosylated peptides by the RP-HPLC analysis were identified as previously described [33].

2.4. Preparation and separation of rFVIIa oligosaccharides

Samples of rFVIIa (1 mg/ml) were dialyzed against 0.1% trifluoroacetic acid, lyophilized and hydrazinolyzed on a GlycoPrep1000 (Oxford GlycoSystems, UK), using standard procedure. The dried oligosaccharide pools were resuspended in water and analyzed, using HPAEC–PAD: HPLC equipment, Waters; column, CarboPac PA100 (250 × 4 mm) with a CarboPac PA100 Guard (50 × 4 mm) (Dionex, USA); column temperature, ambient; detection, pulsed amperometric with pulse potentials and durations 0.05 V/300 ms, 0.80 V/200 ms, −0.06 V/600 ms, working at 1 μA full scale; flow-rate, 1.0 ml/min; gradient, 7.5 m*M* to 150 m*M* sodium acetate in 150 m*M* sodium hydroxide for 50 min.

2.5. Sample preparation prior to CE

A change of the buffer of the rFVIIa samples (1 mg/ml) to 50 m*M* sodium acetate, 5 m*M* calcium chloride, pH 4.8, was done in some of the experiments. Buffer exchange was performed either by dialysis (M_r 8000 cutoff) or size-exclusion chromatography (NAP10 column, Pharmacia, Sweden).

2.6. Capillary electrophoresis–instrumentation and operation

The capillary electrophoresis was performed using a Waters Quanta 4000 capillary electrophoresis system. Fused-silica capillaries (75 μm I.D.; 356 μm O.D.) from Polymicro Technologies (Phoenix, AZ, USA) with a total length of 88 or 45 cm were used. Sample introduction was done by hydrostatic injection (siphoning) for 10 s at the anode. UV detection at 214 nm took place 7.5 cm from the cathodic end of the capillary. To avoid excessive heating of the buffer in the capillary the field strength was chosen so that the current did not exceed 100 μA.

Before analysis the capillary was equilibrated with the separation buffer. Washing with dilute sodium hydroxide between runs had no beneficial effect and was therefore avoided. At chang-

ing from buffer containing putrescine to pure buffer the capillary was washed with 1 *M* HCl followed by 1 *M* NaOH, distilled water and finally equilibrated with the new buffer.

As marker for the electroosmotic flow 0.1% benzylalcohol was used. The buffer solution in the electrode vessels was renewed at least once a day.

3. Results and discussion

3.1. Effect of putrescine on CE of rFVIIa

Inspired by the results achieved using putrescine as a buffer additive for CE of glycoproteins, we tested the effect on rFVIIa. CE analysis of rFVIIa in 100 m*M* phosphate buffer, pH 9.0, containing 0, 5 and 25 m*M* putrescine, respectively, is shown in Fig. 1. In phosphate buffer without putrescine (top panel) rFVIIa migrates as a single symmetrical peak followed by a slightly skewed peak representing the sample buffer glycylglycine. Although a separation of rFVIIa into at least two components is clearly visible using 5 m*M* putrescine (middle panel), optimal resolution requires about 25 m*M* putrescine (bottom panel). Addition of putrescine has two effects on the electrophoretic peak pattern. The EOF is markedly reduced and simultaneously rFVIIa is separated into a cluster of peaks. The reduction of the EOF is most probably due to a partial neutralization of the fused-silica surface of the capillary, which is strongly negative at pH 9.0 due to ionization of the silanol groups. It is noteworthy that the optimum concentration of putrescine is about ten times higher compared to what was found to be optimal for ovalbumin [26] and r-HuEPO [24]. Increasing the concentration of putrescine to 50 m*M* prolongs the migration time of rFVIIa even further without any improvement in the resolution of the peak cluster (not shown). This indicates that the resolution of rFVIIa into several close-eluting peaks is only for a part due to a reduction of EOF. Ion-pair formation between putrescine and negatively charged groups of the glycoprotein probably plays also an important role.

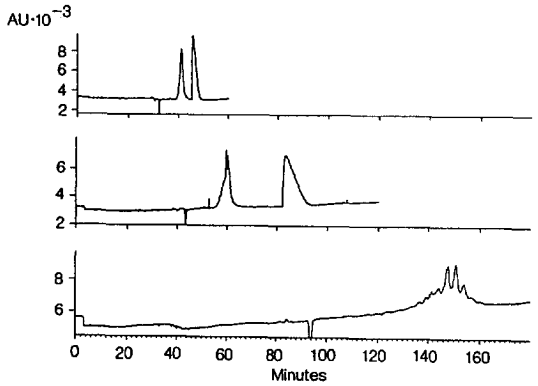

Fig. 1. Capillary electrophoretic separation of rFVIIa glycoforms using putrescine as buffer additive. The separations were performed in an 88-cm long capillary using 100 m*M* phosphate, pH 9.0, and a separation voltage of 10 kV. Top panel: 0 m*M* putrescine; rFVIIa migrates in front of glycylglycine. Middle panel: 5 m*M* putrescine; rFVIIa migrates in front of glycylglycine. Bottom panel: 25 m*M* putrescine.

3.2. Significance of the CE separation buffer substance

The beneficial effect of borate for the resolution of mono- and oligosaccharides as well as glycosylated peptides has been reported [32]. In a study on ovalbumin [26] it was likewise concluded that application of a borate buffer played an important role in the separation of ovalbumin glycoforms.

We have compared the separation of rFVIIa using 100 m*M* phosphate, 25 m*M* putrescine, pH 8.0, respectively 100 m*M* borate, 12.5 m*M* putrescine, pH 8.0, as running buffer. It was necessary to reduce the concentration of putrescine in the borate buffer as the migration time of rFVIIa became too long with 25 m*M*. We observed that replacement of phosphate with borate resulted in longer migration times and the resolution of the peak cluster became worse. We cannot tell, however, whether some complexation between borate and the glycostructures of rFVIIa also takes place. However, it does not improve the separation of the glycoforms. These

observations are in agreement with the findings of Taverna et al. [20] in their studies on rtPA glycopeptides. Further, they support the idea that the resolution induced by addition of putrescine is not only caused by a reduction of the electroosmotic flow but also by ion-pair formation with rFVIIa itself.

3.3. Optimization of the CE separation buffer pH

The influence of buffer pH was investigated in the pH interval 7–9. Optimum separation was achieved around pH 8. At pH ca. 7 the resolution was more or less lost. The reason for this could be that rFVIIa carries a low electrical net charge at this pH. By CE analysis in pure phosphate buffer, pH ca. 7 (without putrescine), rFVIIa behaves in a similar manner (not shown). The separation was not improved when buffer pH was raised to about 9.

3.4. Repeatability of the CE separation of rFVIIa glycoforms

Fig. 2, which shows run nos. 2, 4, 6 and 8 out of eight consecutive runs of the same FVIIa sample, using the same separation buffer, clearly demonstrates that the repeatability of the separation is excellent. However, frequent replacement of the separation buffer was required. With extensive use of the same separation buffer we observed that the resolution gradually became worse. Simultaneously the UV background was increased and the analyte turned yellow. An electrochemical oxidation of putrescine into a six-membered cyclic azo-structure is a possible explanation of these observations.

3.5. Specificity of putrescine

To test whether the separation obtained by adding putrescine to the phosphate buffer was a specific effect of putrescine, we repeated the analysis replacing putrescine with cadaverine (1,5-diaminopentane). Analysis of the rFVIIa sample using respectively putrescine and cadaverine as buffer additive is shown in Fig. 3. Clearly the separation pattern obtained by either of the two additives is essentially the same. This indicates that not only putrescine but probably a larger group of α,ω-diaminoalkanes are potential ion-pair reagents.

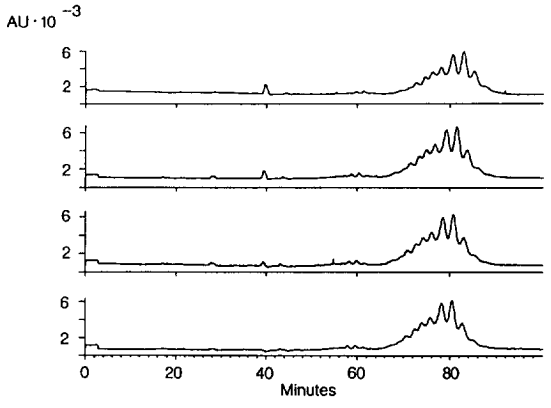

Fig. 2. Repeatability of the separation of rFVIIa glycoforms by capillary electrophoresis. The figure shows from top to bottom analysis nos. 2, 4, 6 and 8 of the same sample. The separation buffer was 100 m*M* phosphate, 25 m*M* putrescine, pH 8.0. A voltage of 4.5 kV was applied on a 45-cm long capillary.

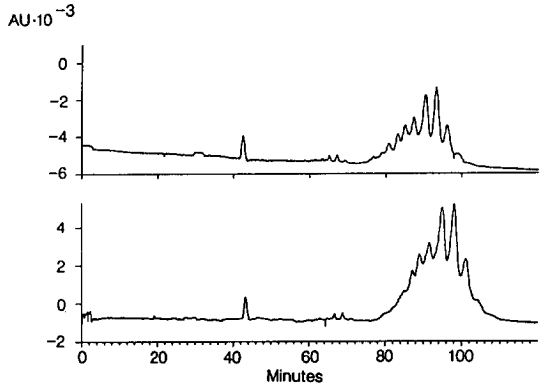

Fig. 3. Separation of rFVIIa glycoforms by capillary electrophoresis in a 45-cm long capillary using 100 m*M* phosphate buffer, pH 8.0, containing either 25 m*M* putrescine (upper panel) or 25 m*M* cadaverine (lower panel) as buffer additive. The separation was performed in a 45-cm long capillary and with a voltage of 4–5 kV.

3.6. CE analysis of native and neuraminidase treated rFVIIa

As the separation in free zone electrophoresis is based upon differences in the mass-to-charge ratio of the analytes, it was assumed that the observed resolution of rFVIIa was related to the different content of Neu5Ac of the individual glycoforms. Studies on rtPA [18] and r-HuEPO [24] supported this assumption. The CE analysis of rFVIIa before and after treatment with neuraminidase is shown in Fig. 4. It is obvious that the removal of Neu5Ac gives a much simpler peak profile of rFVIIa and a shorter migration time than the native protein. By removal of the Neu5Ac residues positioned terminally on the oligosaccharide structures a reduction is obtained in the net negative charge of the individual glycoforms as well as in the charge heterogeneity of the glycoform population. Reduction of the net negative charge leads to an increase in migration rate and reduction in charge heterogeneity results in a peak profile that is much less broad, and both effects are observed for rFVIIa (Fig. 4). This supports the idea that the peak cluster represents glycoforms of rFVIIa having different numbers of Neu5Ac residues. Some heterogeneity of neuraminidase treated rFVIIa is obviously still present. Since extended neuraminidase treatment gave no further change in the peak profile (not shown), it is concluded that the remaining heterogeneity is not caused by incomplete removal of Neu5Ac.

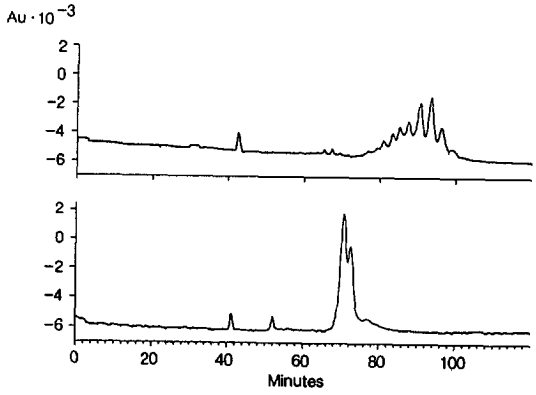

Fig. 4. Capillary electrophoresis of rFVIIa before and after treatment with sialidase. The separation was performed in 100 m*M* phosphate with 25 m*M* putrescine, pH 8.0, using a 45-cm long capillary and a voltage of 5 kV. Top panel: native rFVIIa. Bottom panel: sialidase treated rFVIIa.

3.7. RP-HPLC analysis of tryptic Asn322 glycopeptides from native and from neuraminidase treated rFVIIa

In RP-HPLC of the tryptic peptides from native rFVIIa, the glycopeptides containing the Asn322 glycosylation site elute as several distinct peaks (Fig. 5). These glycopeptide peaks have previously been characterized to be the rFVIIa glycopeptide fragment 317–326 (i.e. Val-Gly-Asp-Ser-Pro-Asn(glycosylated)-Ile-The-Glu-Tyr) and the separation has been shown to be based on the content of Neu5Ac in the oligosaccharide structures linked to the peptide so that with increasing retention time the number of Neu5Ac residues decrease [33]. In comparison, the tryptic Asn322 glycopeptides from neuraminidase treated rFVIIa elute as two peaks only (Fig. 5). Since we have no indication of incomplete removal of Neu5Ac, separation by RP-HPLC analysis of the desialylated glycopeptides is apparently based on other differences in the oligosaccharide structures than the content of Neu5Ac. Similar results have been obtained by RP-HPLC of the Asn145 glycopeptides (not shown).

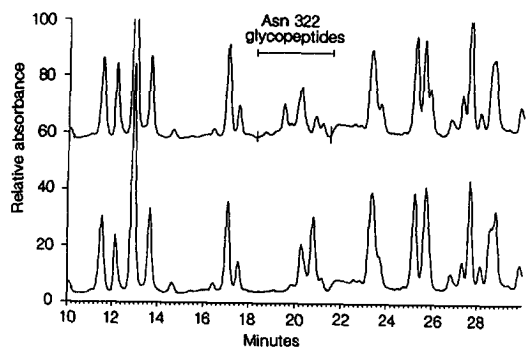

Fig. 5. Reversed-phase HPLC separation of tryptic peptides from native rFVIIa (top) and from neuraminidase treated rFVIIa (bottom). The peaks corresponding to the glycopeptides containing the heavy chain N-glycosylated site Asn322 are marked Asn322 glycopeptides. The separation conditions are described in Section 2.3.