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A sensitive mapping strategy for monitoring the reproducibility of glycan processing in an HIV vaccine, RGP-160, expressed in a mammalian cell line

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The external envelope glycoprotein (gp 160) of HIV-1 is a candidate for vaccines against AIDS. Most of the surface of the molecule is shielded by carbohydrate and the structures and locations of these glycans may be important in defining the immunogenicity of the viral coat. Here we report a sensitive mapping strategy for profiling and analysing the N-glycosylation of gp160, based on chemical release of glycans, fluorescent labelling and HPLC analysis. This approach has been validated in terms of establishing the reproducibility of all steps in the analytical procedure and on overall reproducibility on a run-to-run and day-to-day basis. The validated analysis technique was used to monitor the consistency of N-glycosylation of one rgp 160 vaccine candidate produced in bovine hamster kidney (BHK) cell culture. It was demonstrated that the variation in the glycan profiles of 6 different lots was not statistically significant.

Keywords: **glycan mapping, 2-aminobenzamide, gp160, HIV-1 envelope glycoprotein, normal phase chromatography, weak anion exchange chromatography**

Abbreviations: **2-AB, 2-aminobenzamide; AIDS, Acquired Immune Deficiency Syndrome; BHK, baby hamster kidney; HIV, human immunodeficiency virus; HPLC, high performance liquid chromatography; NP-HPLC, normal phase high performance liquid chromatography; RSD, relative standard deviation; WAX, week anion exchange chromatography.**

Introduction

The glycosylation of recombinant proteins varies according to the cell culture conditions, the nature, the type of the host cells used [1,2] and on the downstream processing techniques used for the isolation of the protein [3]. Accumulating evidence on the role of glycosylation in achieving and maintaining the 3D structure of proteins and the contribution of attached sugars to numerous functions and properties of a protein [4,5,6] has stimulated the development of rapid and reliable methods to analyse the glycosylation of proteins of therapeutic value.

A feature of gp160 is that about 50% of its molecular weight consists of N-linked glycans [7] in addition to mucin type O-linked glycans [8]. This extensive glycosylation suggests that most of the surface of the protein is shielded by large glycans which are mobile, particularly the N-glycans which are flexible in their linkage to asparagine residues [9]. Mammalian cell lines have the glycosylation machinery to produce patterns of glycans similar to that found in humans [10] and in this case a Bovine Hamster Kidney (BHK) cell line was used. Specific functions for such glycans are not yet clear, however the fact that BHK expressed proteins are likely to resemble their natural counterparts in terms of glycosylation may well be significant for vaccine production.

Therefore it is important to characterize the glycans attached to glycoproteins which are potential recombinant vaccines, such as rgp160, on a batch-to-batch basis to assure the consistency of the prototype vaccine production. Clearly the reliability of such quality control depends on the reproducibility of the analytical procedures and the purpose of our investigation was to determine such parameters.

The procedure described here involves the release of neutral and sialylated N-glycans from the glycoprotein using

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automated hydrazinolysis followed by 2-AB labeling of the released oligosaccharides and their subsequent separation by HPLC.

Materials and methods

Reagents and proteins

Ammonium hydroxide and formic acid of HPLC grade were from Sigma (St Louis, USA) and Prolabo (Fontenay-sous bois, France) respectively. Acetonitrile was from Fisher (Maurepas, France). The recombinant soluble gp160s-MN/LAI variant was produced from vaccinia virus-infected BHK cells, isolated from the cell-free supernatant after concentration and diafiltration, and purified by immunoaffinity as described previously [11] (Pasteur Mérieux Connaught, Marcy l'Etoile, France).

Methods

After exhaustive dialysis of the gp 160s-MN/LAI (TFA 0.1% in water, 4 days, 4 °C), glycans were released by hydrazinolysis [12] using automation [13] on the GlycoPrep 1000 (Oxford GlycoSciences, Abingdon, UK) operating in the N-mode (5 h, 95 °C), and samples evaporated to dryness before 2-AB labelling [14] using a signal kit (2 h, 65 °C) from Glyko (Novato, CA).

The HPLC system consisted of a P1000 XR gradient pump (Thermo Seperation Products), a fluorescence detector FP 920 (JASCO) (λ exc = 330 nm and λ emiss = 420 nm). A Shimadzu (CTO-6A) and a WO Industrial Electronics (Jetstream 2) temperature control modules were employed. The labeled glycans were first separated by WAX-chromatography on a GlycoSep C column (4.6 × 100 mm) (Glyko, Novato, CA) using a 0.4 ml min^{-1} flow rate. Conditions used for the gradient were: Solvent A (acetonitrile), solvent B (water) and solvent C (ammonium acetate buffer. 500 mM, pH 4.5). Initial conditions were 75% A, 25% B, for 5 min, followed by a linear gradient of 75–65% A and 25–35% B over 15 min, followed by 65–10% A and 35–38% B and 0–52% C over 30 min, and finally followed by 10–0% A and 38–40% B and 52–60% C over 10 min, returning to the start conditions over the next 1 min. Column temperature was 35°C.

The 2-AB glycans were also separated by NP-chromatography on a GlycoSep N column (4.6 × 250 mm) (Glyko, Novato, CA) using the following gradient conditions: Solvent A (acetonitrile), solvent B (ammonium formate, 50 mM, pH 4.5). Initial conditions were 65% A at a flow rate of 0.4 ml min^{-1}, followed by a linear gradient of 65–50% A over 70 min, followed by 50–0% A over 1 min. The column was washed in 100% B for 9 min before decreasing the concentration to 65% A. Column temperature was 30 °C.

Glycan mapping was performed by injecting a glycan pool corresponding to 2 to 4 μg of the starting glycoprotein.

Results and discussion

The analysis of the glycans requires that they are first released from the peptide. In this case chemical release by hydrazinolysis was chosen as this has been shown to non-selectively release all glycans. It does however require use of hazardous chemicals and enzymatic release is often used as an alternative. In the case of rgp160 a comparison of two release techniques has been performed and will be reported elsewhere [15]. The labelling enables sensitive and quantitative detection of glycans and 2-amino benzamide was chosen as the label is non-selective and has been validated for sialylated structures.

Repeatability and intermediate precision of the analytical methods

The released and derivatized glycans were analysed by WAX- and NP-chromatography (Figure 1). The performance of the two chromatographic methods in terms of intra-day and inter-day reproducibility of both retention times and relative peak areas have been evaluated. Intra-day reproducibility was estimated from 6 replicate analyses of the same glycan pool while the inter-day reproducibility was determined for three consecutive days.

For WAX analysis, peaks corresponding to the charged glycans were not fully resolved so that individual peaks could not be integrated accurately. As shown in Table 1, apart from the less abundant species Na (which represented less than 0.3% of the total) inter- and intra-day reproducibility of the relative peak areas was quite satisfactory (RSD < 5.6%). For the amide stationary phase, 9 peaks were taken into account for these calculations. Peaks P4 and P5 were integrated as one. Table 2 shows the RSDs calculated for retention times and percentage areas of the peaks. Very satisfactory inter- (RSD < 3.6%) and intra-day (RSD < 1.6%) reproducibility was obtained for the relative peak areas. In addition, retention times were highly reproducible with RSDs not exceeding 1.2%. Although the amide column separates the glycan pool into fewer peaks, the high reproducibility makes this method particularly suitable for glycan mapping and studies of the lot-to-lot consistency of the glycosylation of recombinant protein.

Reproducibility of the glycan release and derivatization

On the basis of the results obtained above, NP-HPLC analysis was selected as the most appropriate technique to compare the reproducibility of the preliminary stages of the preparation of the derivatized glycans. The reproducibility of the automated hydrazinolysis reaction was determined by performing 6 different releases of the same lot of rgp 160. Reproducibility of the derivatization step was determined by labelling six aliquots of the same glycan pool with 2-AB.

The reproducibility of these two steps was evaluated in terms of relative peak areas as previously described. The

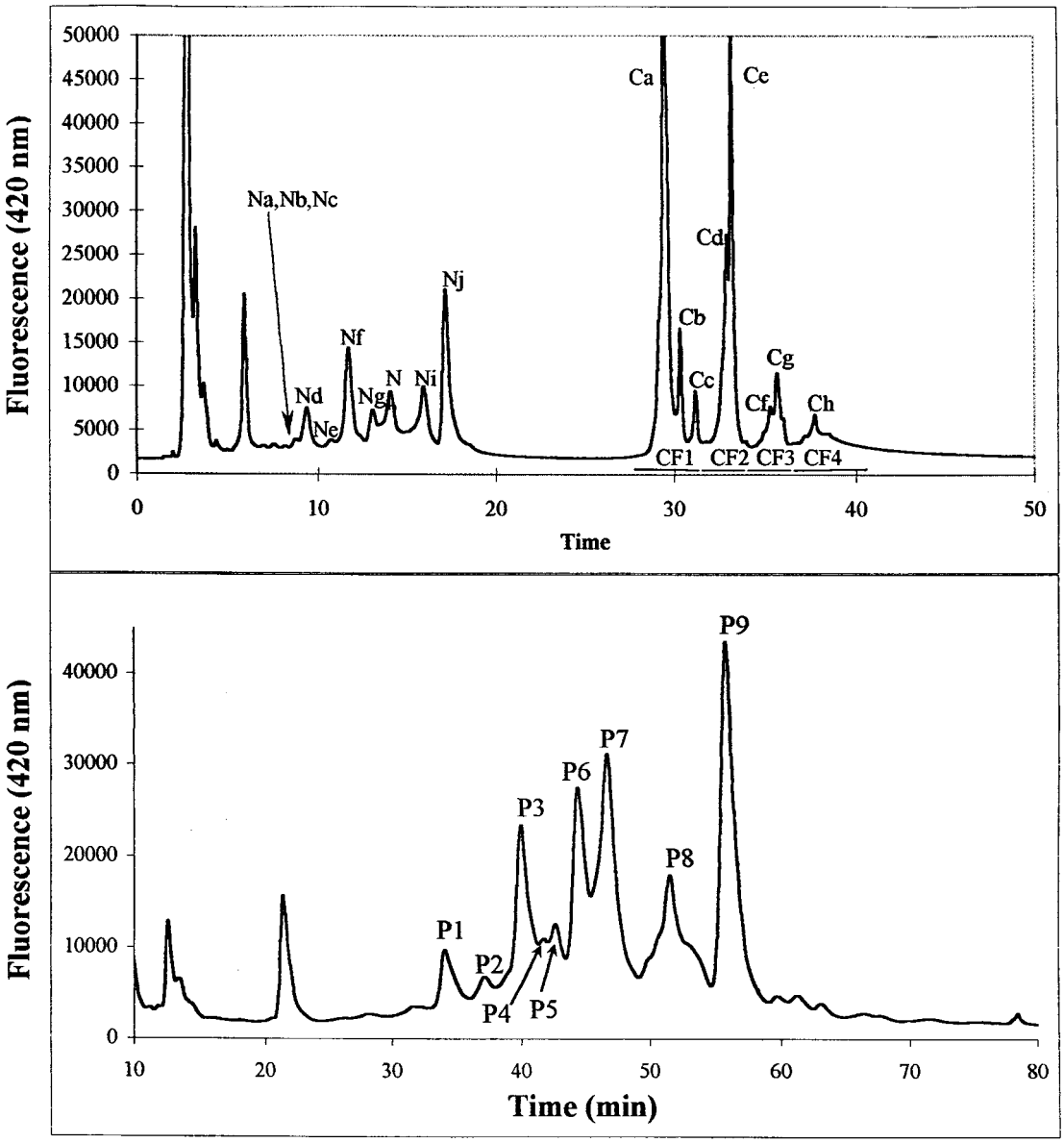

Figure 1. Weak anion exchange (A) and Normal phase (B) chromatography of the glycans released from rgp160s following hydrazinolysis of 5 μg of glycoprotein. The chromatogram obtained in (A) is divided into two main regions, the first (5–20 min) corresponds to the elution positions of neutral glycans (Na to Nj) which eluted in an approximately increasing order of molecular weight. The second region (27–40 min) which represents the charged glycans eluting in the order of increasing charge is split into four main fractions corresponding to structures having from one to four negative charges. For conditions see Materials and methods.

derivatization step was highly reproducible, with RSDs of the relative peak areas for P1 to P9 less than 1.0%. In contrast, we observed relatively high RSD for the reproducibility of the automated hydrazinolysis of the samples (RSD from 2% to 10%). The hydrazinolysis step includes variations arising from the variable yields of both the release of glycans and the derivatization steps as well as those from the chromatographic analyses. The RSD values calculated for 6 independent hydrazinolyses of the same lot of rgp160 reflect the variation of the results that may be expected for a given glycoprotein pool submitted to all procedures.

Lot-to-lot consistency of the glycosylation

In view of the results obtained for the reproducibility of the hydrazinolysis step, we analysed the lot-to-lot consistency of the glycosylation by performing the complete procedure (hydrazinolysis, derivatization with 2-AB and NP-HPLC

Table 1. Inter-day (n = 3) and intra-day precision (n = 6) of the GlycoSep C chromatographic method.

	Migration times			*Peak areas*		
Peaks	*Intraday RSD*	*Interday RSD*	*Peaks*	*Relative peak area (%)*	*Intraday RSD*	*Interday RSD*
Na[1]	1.94	4.15	Na[1]	0.34	7.87	3.36
Nb	2.13	5.32	Nb	0.46	5.15	2.14
Nc	2.05	5.4	Nc	1.27	3.55	1.86
Nd	1.95	5.21	Nd	6.94	1.34	0.67
Ne	1.92	5.63	Ne	1.70	3.31	1.35
Nf	1.77	5.26	Nf	19.29	1.05	2.28
Ng	1.76	5.16	Ng	6.94	3.26	5.58
Nh	1.48	4.39	Nh	14.24	3.07	2.27
Ni	1.82	3.71	Ni	15.35	0.98	3.92
Nj	1.07	3.38	Nj	33.52	1.81	3.22
Ca[2]	0.11	0.41	CF1[2]	53.65	0.85	1.85
Cb	0.10	0.41	CF2	33.88	1.44	2.01
Cc	0.09	0.55	CF3	8.58	2.63	2.38
Cd	0.18	0.09	CF4	3.88	5.51	3.5
Ce	0.15	0.09				
Cf	0.16	0.07				
Cg	0.18	0.33				
Ch	0.15	0.07				

[1]Relative peak areas represent the ratio of the area of each peak (Na to Nj) obtained for neutral structures on the sum of the peak areas of the neutral structures.
[2]Relative peak areas represent the ratio of the area of each peak (Ca to Ch) or each group of peaks (CF1 to CF4) obtained for charged structures on the sum of the peak areas or the areas of the group of peaks of the charged structures.

Table 2. Inter-day (n = 3) and intra-day precision (n = 6) of the GlycoSep N chromatographic method.

	Migration times			*Peak areas*		
Peaks	*Intraday RSD*	*Interday RSD*	*Peaks*	*Relative peak areas*	*Intraday RSD*	*Interday RSD*
P1[1]	0.77	1.06	P1[1]	3.90	0.34	1.03
P2	0.86	1.20	P2	1.68	1.57	3.62
P3	0.79	1.08	P3	13.44	0.47	0.51
P4	0.82	1.12	P4 + P5	6.36	1.05	1.85
P5	0.78	1.01	P6	13.94	0.41	0.96
P6	0.77	1.01	P7	20.58	0.65	1.07
P7	0.76	1.00	P8	13.72	0.64	1.07
P8	0.61	0.76	P9	26.37	0.13	0.37
P9	0.60	0.70				

[1]Relative peak areas represent the ratio of peak area (P1 to P9) on the sum of the peak areas.

Table 3. Lot-to-lot reproducibility study of the relative peaks areas of the main chromatographic peaks (P1 to P9) obtained by NP-HPLC.

	Among groups (n = 6)[1]		*Within groups (n = 6)*		
Peaks	*RSD (%)*	*Variances*	*Relative peak areas*	*RSD (%)*	*Variances*
P1[2]	9.9	0.33	4.31	13.5	0.34
P2	15.8	0.13	2.15	15.2	0.11
P3	3.9	0.35	14.13	8.5	1.44
P4 + P5	5.8	0.08	6.45	8.5	0.30
P6	1.9	0.15	14.30	3.9	0.32
P7	8.0	1.00	20.50	7.4	2.30
P8	3.4	0.20	12.50	9.3	1.36
P9	5.4	2.13	25.67	5.3	1.84

[1]The among group variances were estimated from the hydrazinolysis variances.
[2]Relative peak areas were calculated as described in Table 2.

separation of the glycans) for six different production lots (C, E, F, G, H and K). Chromatographic analyses of the released glycans from each of the 6 different lots were performed in triplicate at day 1, day 2 and day 3. The same chromatographic criteria as those described previously were determined (relative peak areas of P1 to P9) and the average of the three relative peak areas were calculated for each (Figure 2).

Analysis of variance

To carry out an accurate comparison of the glycosylation of the different lots, we compared among-group variance with the within-group variance obtained in 6 different lots for the relative areas of the peaks using a Fisher t test. The among-group variance was estimated from the results obtained for the reproducibility study of hydrazinolysis. As shown in Table 3, probabilities are greater than 0.05 except for the peak P8 exhibiting a probability above 0.025. These results indicate that the apparent differences between the 6 different lots is not statistically significant at a 97.5% confidence level.

Conclusion

In order for a glycan analysis technique to be accepted in routine use the reproducibility of the techniques must be measured and fully validated in terms of its ability to separate glycans. Unfortunately this has been difficult in glycan analysis and this has restricted complete analysis of glycans in production batches of glycoproteins. The information may also be required to establish the identity of the product for patenting purposes. Although several techniques have been described for routine glycan analysis, in many cases the reproducibility is not known nor structures can be completely identified from the profiles. In the technique described here high reproducibility has been demonstrated and it is possible to characterise each peak [15]. The high reproducibility of the NP-HPLC method is particularly suitable for glycan mapping and determining the lot-to-lot consistency of the glycosylation of recombinant proteins. Moreover, good reproducibility was demonstrated for the derivatization step using 2-AB. Examination of the three stages of the procedure (release, derivatization with 2-AB and NP-HPLC of the glycans) indicated that the major source of variation in this procedure is the automated hydrazinolysis step.

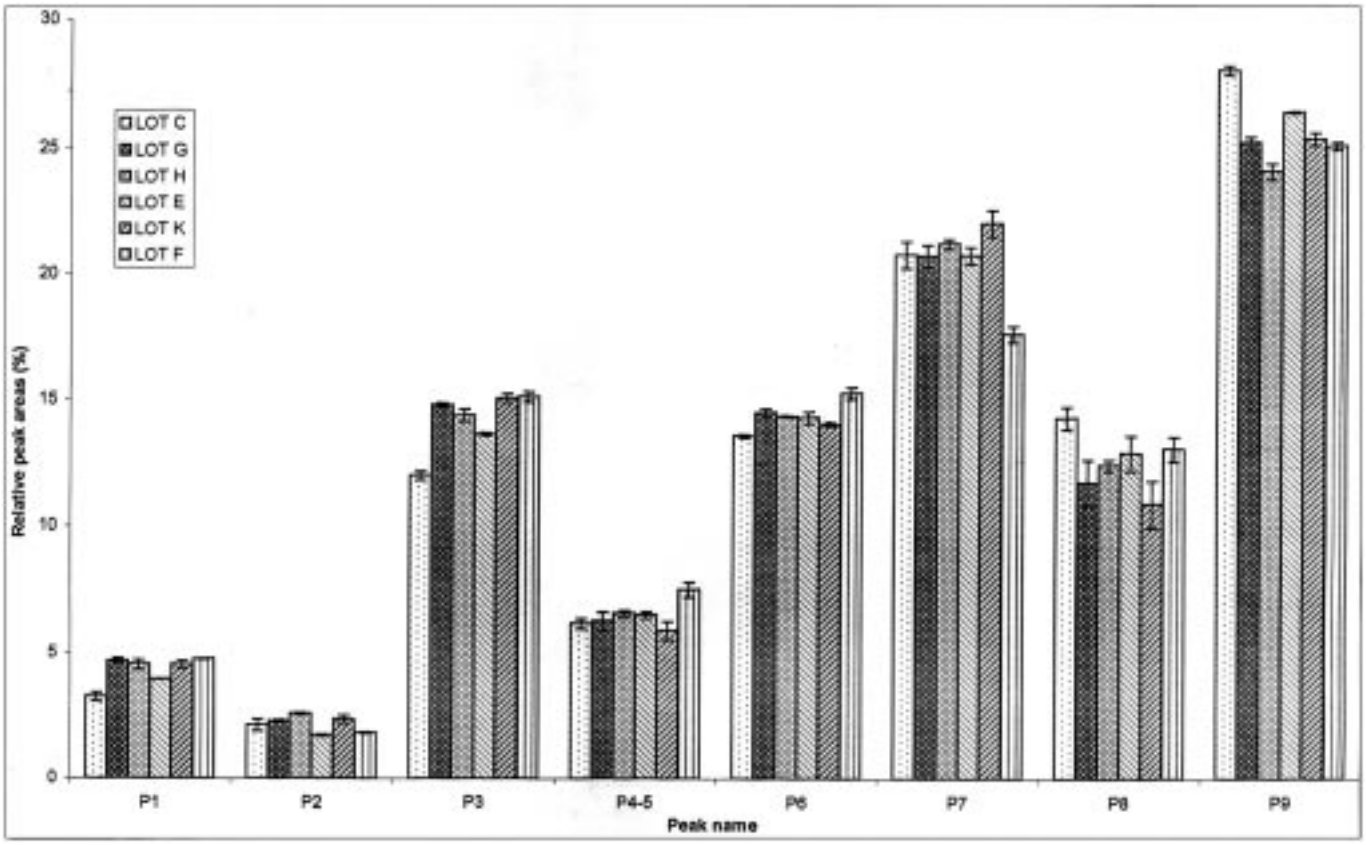

Figure 2. Histograms representing the lot-to-lot reproducibility study of the relative area of the main chromatographic peaks obtained by NP-HPLC for 6 different lots. Values represent the means ± SD (for 3 consecutive chromatographic analyses).

We propose that such a validated procedure using a combination of HPLC analyses can be applied in a routine manner to give reliable and informative data on recombinant glycoproteins destined for vaccination or therapy.

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