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A Strategy for the Mapping of *N*-Glycans by High-pH Anion-Exchange Chromatography with Pulsed Amperometric Detection[1,2]

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We have evaluated the high-pH anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) with respect to its suitability to establish a carbohydrate mapping database that would enable carbohydrate structural analysis by mere comparison of retention times. The suitability of HPAE-PAD for carbohydrate structural analysis was ascertained by validation experiments. The retention times of distinct *N*-glycans, prepared and measured on different days, were shown to be highly reproducible, with a coefficient of variation (CV) of <0.5%, requiring less than 100 pmol of *N*-glycan per injection for reliable measurements. Including appropriate internal chromatographic standards, such as (Neu5Ac)1, (Neu5Ac)2, (Neu5Ac)3, and Neu5Gc, the HPAE-PAD method fulfills the analytical requirements with respect to accuracy, precision, reproducibility, and sensitivity. The *N*-glycan mapping database was established, using two optimized linear gradients "S" and "A" for sialylated and asialo *N*-glycans, respectively. Approximately 100 different *N*-glycans of known structure, which have thus far been measured and characterized, have entered our Lotus 1–2–3 mapping database. The efficiency of the database for structural determinations was tested, using the *N*-linked carbohydrates isolated from rhuEPO, expressed in BHK cells. Nine different sialylated *N*-glycans of rhuEPO (BHK) could be assigned with a deviation of <±0.5%, using gradient S, and six of the eight asialo *N*-glycans of rhuEPO (BHK) detected with gradient A could be assigned with an accuracy of <±1%, three of them even with an accuracy of <0.1%, proving the reliability of the established HPAE-PAD mapping database.

The demand for reproducible, fast, and facile carbohydrate analysis is increasing steadily. However, the structural analysis of complex carbohydrates requires expertise and infrastructure for methylation analysis/GC-MS, FAB[4]-MS, and high-resolution ^{1}H-NMR spectroscopy. Therefore, an alternative that would allow one to reduce these high-cost and time-consuming demands of carbohydrate structural analysis by using standard separation techniques and affordable hardware is attractive.

High-pH anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) (1) appears to fulfill the criteria for such a purpose. It allows oligosaccharide separation and detection without requiring prior derivatization and appears therefore supe-

[1] A preliminary report was presented at the 11th International Symposium on Glycoconjugates, June 30–July 5, 1991, Toronto, Ontario, Canada; Abstracts of Papers, *Glycoconjugate J.* **8**, 248, Abstract 14.3 (1991).

[2] Dedicated to Professor Dr. Hans Paulsen, University of Hamburg, on the occasion of his 70th birthday.

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[4] Abbreviations used: FAB, fast atom bombardment; HPAEC, high-pH anion-exchange chromatography; PAD, pulsed amperometric detection; RT, retention time; CV, coefficient of variation; PA, pyridylaminated; Neu5Ac and (Neu5Ac)1, *N*-acetylneuraminic acid; (Neu5Ac)2, Neu5Ac α2,8Neu5Ac; (Neu5Ac)3, Neu5Ac α2,8Neu5Ac α2,8Neu5Ac; Neu5Gc, *N*-glycolylneuraminic acid; S, sialic acid; G and Gal, D-galactose; N and GlcNAc, *N*-acetyl-D-glucosamine; M and Man, D-mannose; F and Fuc, L-fucose; LacNAc, *N*-acetyllactosamine; rhuEPO, recombinant human urinary erythropoietin; BHK, baby hamster kidney; PA, pyridylaminated; t-PA, tissue plasminogen activator; PNGase F, polypeptide: *N*-glycosidase F.

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rior to the existing two-dimensional mapping technique (2) that needs the introduction of a fluorescent (2-aminopyridyl) label. The simple chromatographic procedure could be shown to be applicable to routine bath analysis of biotechnologically prepared glycoproteins such as EPO (3–5), antithrombin III, *t*-PA, and interferon-β from several recombinant host cell lines (Conradt *et al.*, unpublished data). It is highly reproducible and superior to conventional chromatographic techniques, requires only tiny amounts of material in the picamole range (6), and does not need expensive instrumentation (GC-MS, FAB-MS, NMR), which is required for primary structural analysis of oligosaccharides derived from glycoproteins (7).

In order to initiate the desired database, approximately 60 different *N*-glycans were isolated from purified glycoproteins or tryptic digests thereof according to known procedures, and their structures were determined by methylation analysis, FAB-MS, and ^{1}H-NMR spectroscopy (4,5,7–14). The corresponding asialo *N*-glycans were obtained by neuraminidase treatment. Sequential exoglycosidase digests (of known specificities) of distinct asialo structures were performed, providing further *N*-glycans of defined structures. Additional *N*-glycans were purchased, as available. About 100 *N*-glycans have thus far entered the HPAE-PAD mapping database. The efficiency of the database was examined by the structural assignment of the sialylated and desialylated *N*-glycans of rhuEPO (BHK).

TABLE 1

HPAEC Eluents and Gradients D, S, and A

Gradient	Min.	Eluent 1	Eluent 2
		0.1 M NaOH	0.6 M NaAc in 0.1 M NaOH
D	0	100	0
	2	100	0
	20	80	20
	25	70	30
	30	70	30
	50	0	100
	55	0	100
S	0	100	0
	2	100	0
	50	65	35
	57	30	70
	60	0	100
	63	0	100
	64	100	0
	70	100	0
		0.2 M NaOH	0.6 M NaAc in 0.2 M NaOH
A	0	100	0
	5	100	0
	35	80	20
	45	0	100
	50	0	100
	51	100	0
	60	100	0

EXPERIMENTAL

HPAE-PAD Apparatus and Conditions

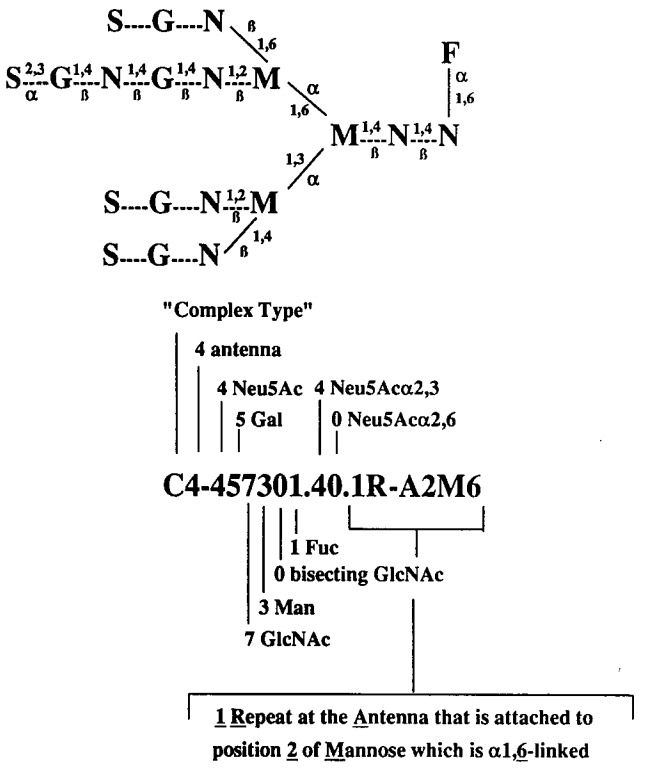
Oligosaccharide mapping was performed with the setup previously described (15), using the Dionex BioLC system (Dionex, Sunnyvale, CA) equipped with a CarboPac PA-100 column (4.6 × 250 mm) and a PA-100 guard column, working at a flow rate of 1 ml/min at ambient temperature. Pulse potentials and durations were 0.05 V/300 ms, 0.60 V/120 ms, −0.60 V/60 ms, working at 300 nA full scale. Sample injection was performed automatically, using a Gilson/Abimed Model 231 sample injector in which the factory-installed rotor seal was replaced with a Tefzel seal to withstand the alkalinity of the eluents. The sample injector was equipped with a Model 401 dilutor and a 20-μl sample loop. The samples (40 μl each) were placed in 100-μl microvials (Hewlett–Packard). The sampling by the Gilson/Abimed sample injector/dilutor was as follows: sample suction, 35 μl each; sample injections, 30 μl each. The elution of the oligosaccharides was performed using the eluents and gradients outlined in Table 1. The discontinuous gradient "D" was initially used for research purposes, but was later replaced by the two optimized linear gradients "S" (which is preferably used for sialylated structures) and "A" (for asialo structures). Gradients S and A were used to initiate the mapping database. The resulting chromatographic data were integrated and plotted using the Dionex AI-450 Model II "full-control" software 3.01/interphase for PC.

Oligosaccharides

N-Glycans were liberated from recombinant glycoproteins by PNGase F treatment and isolated by FPLC/Mono-Q (Pharmacia, Uppsala, Sweden), followed by HPLC on an NH_2-bonded phase (Lichrosorb-NH_2, Merck, Darmstadt, Germany) (4,8). The detailed structural analysis of these *N*-linked oligosaccharides was performed by GC-MS (methylation and compositional analysis), FAB-MS, and ^{1}H-NMR spectroscopy (4,5,7–14). Exoglycosidase digestions were performed as previously described (14). Other *N*-glycans were purchased from Oxford GlycoSystems (Oxon, England) or from Dionex, as available.

Internal Standards

Neu5Ac and Neu5Gc were purchased from Sigma. (Neu5Ac)2 and (Neu5Ac)3 were prepared from colominic acid (Sigma) by hydrolysis in 0.1 N sulfuric acid, 2 h at 80°C, followed by neutralization with 0.4 N sodium



SCHEME 1. Proposed nomenclature of *N*-glycans, depicted by example.

hydroxide. The hydrolysate was desalted (Sephadex G-25, superfine) and fractionated via Mono-Q (Pharmacia), and each fraction was desalted again (Sephadex G-25, superfine). The fractions were analyzed by HPAE-PAD.

Analysis of the N-Glycans of rhuEPO (BHK)

rhuEPO (BHK) was from Merckle GmbH (Ulm, Germany) and represents a pharmaceutically developed product of Elanex Pharmaceuticals (Bothell, WA). The liberation by PNGase F (Boehringer-Mannheim GmbH, Penzberg, Germany) of the EPO *N*-glycans and the desialylation by *Vibrio cholerae* neuraminidase (Behringwerke AG, Marburg, Germany) of the isolated EPO *N*-glycan pool was as described by Conradt and colleagues (5).

Numbering of N-Glycans

The nomenclature of the *N*-glycans was adapted from Oxford GlycoSystems' product catalogue, which corresponds to that commonly used in the glycobiology literature. However, in order to allow proper search profiles in the Lotus 1–2–3 mapping database, the nomenclature had to be extended and improved, as illustrated for complex type *N*-glycans in Scheme 1.

Prefixes M, H, and C indicate the class, i.e., high-mannose, hybrid, or complex-type structures, respectively. C2–, C3–, C4–, and C5– indicate the antennarity of the common complex-type structure:

C2 = biantennary
C3 = triantennary
C4 = tetraantennary
C5 = pentaantennary.

The next four digits following the hyphen, reading from left to right, describe the number of residues of each of the following monosaccharides, respectively:

position 1 = sialic acid
position 2 = galactose
position 3 = *N*-acetylglucosamine, excluding bisecting GlcNAc
position 4 = mannose.

Position 5 is either 1 or 0 and indicates the presence (=1) or absence (=0) of bisecting GlcNAc. Position 6 is either 1 or 0 and indicates the presence (=1) or absence (=0) of Fuc α1,6 at the proximal GlcNAc.

For sialylated structures, the digits are followed by a point. The next two digits following the point relate to the sialic acid linkage: The first digit following the point indicates the number of sialic acid residues α2,3-linked to Gal; the second digit following the point indicates the number of sialic acid residues α2,6-linked to Gal. In asialo structures the theoretical suffix ".00" is omitted.

Thus, for example, the biantennary complex-type *N*-glycan from human transferrin reads C2-224300.02; the corresponding asialo structure reads C2-024300. The tetraantennary fucosylated structure of recombinant erythropoietin from Chinese hamster ovary or BHK cells reads C4-446301.40; the corresponding asialo structure reads C4-046301.

Points generally mark a distinct set of information.

Thus, it appears feasible, for example, to indicate alditol structures by the suffix ".ol" or structures liberated by treatment with endo-H (concomittant with the cleavage of the chitobiose disaccharide unit of the core structure) by the suffix ".endo-H."

If, for example, a LacNAc repeat (R) is located at the antenna (A) that is attached to position 2 of the mannose unit that is α-linked to position 6 of the β-linked mannose unit, the information reads ".1R-A2M6" (Scheme 1). If the repeat is present in the antenna that is attached to position 4 of the mannose unit that is α-linked to position 3 of the β-linked mannose unit, the information reads ".1R-A4M3" (Scheme 1). If two repeats are located at the same antenna, e.g., "A2M6," the information reads ".2R-A2M6" (Scheme 1). A Gal α1,3 unit attached to OH-3 of β-Gal, located, e.g., on A2M6,

TABLE 2

Validation Experiments with and without Neu5Ac as Internal Standard, Measured with Gradient *D*

Sample	RT-Neu5Ac	RT-Pr	RT-Pr-Corr	No. of Runs	Remarks	Remarks
Neu5Ac	16.15			19	Average (min)	
	0.06				SD (min)	
	0.39%				Variation (CV)	
C4-446301.40		32.31		6	Average (min)	Without internal standard
		0.12			SD (min)	
		0.37%			Variation (CV)	
	16.25	32.36	32.16	1		
C4-046301	16.13	14.54	14.56	8	Average (min)	
	0.03	0.06	0.04		SD (min)	
	0.20%	0.41%	0.27%		Variation (CV)	
	15.38	13.80	14.49	7	Average (min)	Helium pressure drop
	0.60	0.52	0.06		SD (min)	
	3.91%	3.76%	0.41%		Variation (CV)	
C4-057301.1R-A2M6		14.99		7	Average (min)	Without internal standard
		0.05			SD (min)	
		0.32%			Variation (CV)	
	15.89	14.75	14.99	11	Average (min)	Helium pressure drop
	0.38	0.37	0.05		SD (min)	
	2.38%	2.54%	0.36%		Variation (CV)	

Note. RT-Neu5Ac, retention time of sialic acid; RT-Pr, retention time of the product; TR-Pr-Corr, retention time of the product, corrected against Neu5Ac as internal standard.

reads "G3-A2M6," where "G3" indicates the linkage position. Similarly, α2,3- and α2,6-linked sialic acid residues are indicated by the suffix "S3-A2M6" or "S6-A2M6," S3 and S6 again indicating the respective linkage position.

In triantennary structures the two isomeric forms are distinguished by the suffix A4M3 or A6M6.

RESULTS

Validation Experiments, Using Gradient D (Table 1)

In the originally used discontinuous gradient D, the retention time of Neu5Ac was 16.15 ± 0.06 min (CV 0.39%) (average of 19 runs on different days; Table 2). The retention time of C4-446301.40 without internal standard was 32.31 ± 0.12 min (CV 0.37%) (average of 6 runs on 2 different days). This value was confirmed, using Neu5Ac as internal standard (Table 2). The retention time of C4-046301 with Neu5Ac as internal standard was 14.56 ± 0.04 min (CV 0.27%) (average of 8 runs on 2 different days) (Table 2).

In a distinct series of runs of C4-046301 with Neu5Ac as internal standard, the helium used to sparge and pressurize the eluents was turned off. Therefore, a dramatic decrease of the retention time of Neu5Ac (down to 14.15 min) was observed. In these runs the CV of the measured samples was increased to 3.76%, while CV of the corrected retention times was 0.41% (7 runs on a single day; Table 2). These results demonstrate the necessity of the internal standard as well as the efficiency of the correction.

The retention time of C4-057301.1R-A2M6 without internal standard was 14.99 ± 0.05 min (CV 0.32%) (average of 7 runs on 3 different days). In the runs with helium pressure drop, the same retention time was achieved (14.99 ± 0.05 min; CV 0.36%; average of 11 runs on 3 different days), however, only after the retention time of the product (average of 14.75 ± 0.37 min; CV 2.54%) was corrected via the signal of the internal standard, for each (Table 2).

Optimization of the Separation by HPAE-PAD

Two linear gradients S and A were developed for the optimal separation of sialylated and asialo *N*-glycans, respectively. Gradient S was optimized by using a panel of approximately 40 different sialylated *N*-glycans (not shown). Gradient A was optimized similarly by using the corresponding panel of asialo *N*-glycans, obtained from the sialylated *N*-glycan pool by *Vibrio cholerae* neuraminidase treatment (not shown). The optimized gradients S and A, which are presented in Table 1, were used to establish the mapping database. The retention times of the internal standards used in gradients D, S, and A are summarized in Table 3.

TABLE 3

Standard Retention Times of the Internal Standards Used

Internal standard	Gradient	Average RT (min)	SD (min)	CV (%)	No. of runs
(Neu5Ac) 1	*D*	16.15	0.06	0.39	19
	S	16.61	0.06	0.34	27
	A	12.83	0.07	0.56	100
(Neu5Ac) 2	*S*	30.38	0.09	0.28	27
(Neu5Ac) 3	*S*	49.83	0.05	0.10	27
Neu5Gc	*A*	33.72	0.06	0.18	100

The N-Glycan Mapping Database

An extract of 48 *N*-glycans of the HPAEC mapping database, measured with gradient S, is presented in Fig. 1. Another extract of 15 asialo *N*-glycans of the HPAEC mapping database, measured with gradient A, is shown in Fig. 2. The retention times of the structures were corrected against the internal standards, the retention times of which are average values of multiple runs at different days, which are summarized in Table 3. For a better resolution and presentation of the data, the corrected retention times of the structures (*x*-axis) are plotted against the theoretical number of sugar residues (*y*-axis) (Figs. 1 and 2), derived from a detailed structural analysis (4,5,7–14).

Rules for Structural Assignments and Alterations of N-Glycans

The *N*-glycan structures were clearly separated according to their charge, i.e., their number of sialic acid residues, exhibiting retention times in the range of 13–18 min (for asialo), 20–23 min (for monosialylated) 27–32 min (for disialylated), 34–38 min (for trisialylated), and 40–43 min (for tetrasialylated structures), using standard gradient S (Fig. 1).

Structural sialyl isomers carrying Neu5Ac α2,6-linked to Gal eluted earlier than the corresponding isomers in which one or two of the Neu5Ac residues were α2,3-linked to Gal.

Removal of one, two, three, or four α2,3-linked sialic acid residues from C4-446301.40 was found to decrease the retention time of the glycan by 6.5, 13, 19.5, and 26 min, respectively (in gradient S).

The introduction of one or two LacNAc repeats into C4-446301.40 was found to decrease the retention time on the order of 0.8 and 1.7 min, respectively, while the retention time of the corresponding asialo *N*-glycan was increased on the order of 0.7 and 1.4 min (in gradient S) and 1.6 and 3.0 min, respectively (in gradient A) (see also (Fig. 2, upward arrows). Thus, the better resolution of gradient A for asialo structures is obvious. In analogy, introduction of a Gal α1,3 unit into asialo structures (attached to OH-3 of β-Gal residues) resulted in retention times increased on the order of 2 min (in gradient A).

The exchange of a single Neu5Ac by Neu5Gc in C2-224300.02 increased the retention time by 7.7 min (measured with gradient S). The introduction into C2-224301.02 of a sulfate group to OH-6 of the GlcNAc of A2M3 (termed as C2-224301.02.SO4-6N-A2M3) dramatically increased the retention time by 24.0 min, measured in gradient S, and the introduction of the sulfate group into the corresponding asialo structure (C2-024301 → C2-024301.SO4-6N-A2M3) analogously

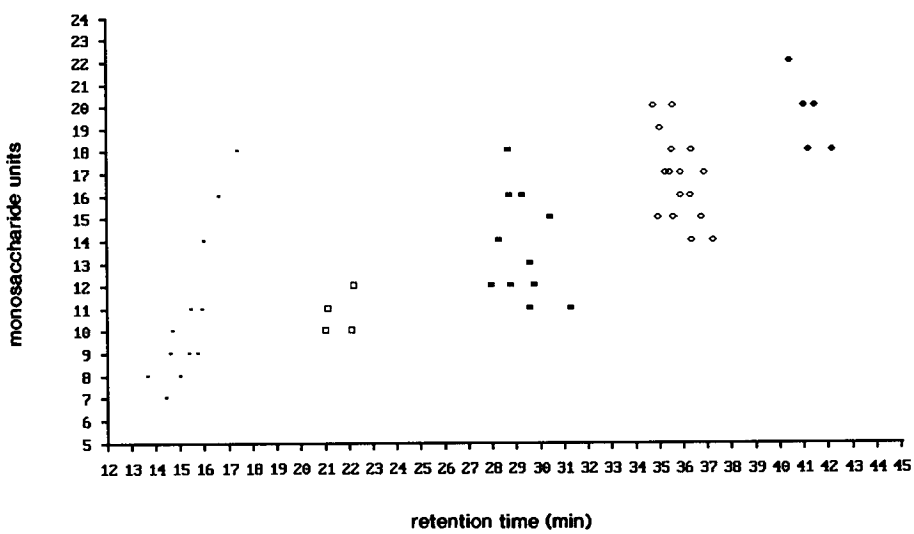


FIG. 1. Database extract of 48 *N*-glycans, measured with gradient S. For better resolution, the corrected retention times of the *N*-glycans (*x*-axis) are plotted against the theoretical number of sugar residues (*y*-axis). •, asialo structures (12); □, monosialo structures (4); ■, disialo structures (11); ◇, trisialo structures (16); ♦ tetrasialo structures (5).

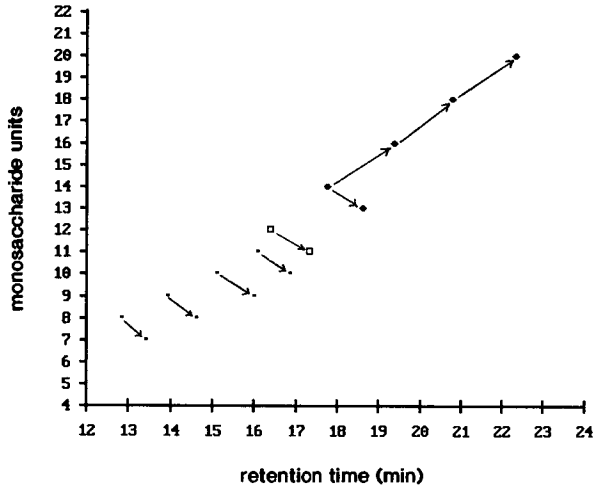


FIG. 2. Database extract of 15 asialo *N*-glycans, measured with gradient A. For better resolution, the corrected retention times of the *N*-glycans (*x*-axis) are plotted against the theoretical number of sugar residues (*y*-axis). Downward arrows indicate shifts achieved upon defucosylation. Upward arrows indicate shifts achieved upon the introduction into C4-046301 of one, two, and three LacNAc repeats. ♦, C4-0 (tetraantennary) structures; □, C3-0 (triantennary) structures; •, C2-0 (biantennary) structures.

increased the retention time by 25.2 min, measured in gradient A.

The removal of Fucα1,6, attached to the proximal GlcNAc, resulted in an increase of the retention time by 1.60 min for C2-224301.02, 0.95 min for C2-124301.01.S6-A2M3, 0.65 min for C2-024301, 0.75 min for C2-004301, and 0.51 min for C2-002301, each measured in gradient S. The removal of Fuc α1,6, as measured in gradient A, is schematically outlined in Fig. 2 (downward arrows).

An increase of the retention times by 0.7–1.0 min was observed upon the introduction of the bisecting GlcNAc into asialo-biantennary *N*-glycans (in both gradients S and A).

The treatment of the biantennary *N*-glycans C2-024301 and C2-024300 with β-galactosidase led to a decrease of the retention times for the corresponding asialo–agalacto structures, C2-004301 and C2-004300, of about 2.6 min each, and the treatment of these structures with hexosaminidase further decreased the retention times of the corresponding structures, C2-002301 and C2-002300, by about 6.3 min each (measured with gradient A).

The Neu5Ac α2,3-linked trisialo-triantennary fucosylated isomers (C3-335301.30.A4M3 and C3-3353-01.30.A6M6) were separated in gradient S by only 0.06 min (· · · A4M3 < · · · A6M6), which is regarded as not significant. However, the corresponding asialo form C3-035301.A4M3 eluted approx. 0.6 min earlier than the C3-035301.A6M6 isomer, using gradient A.

The retention time of asialo structures increased with the number of antennae attached to the core (see also Fig. 2).

Structural Assignment of the N-Glycans of rhuEPO (BHK)

The PNGase F-derived *N*-glycan pool of rhuEPO (BHK) was measured by gradient S (Fig. 3), using Neu5Ac as internal standard (peak 1). After correction of the RT of each peak via the RT of the internal standard, the structures of 9 of the 15 peaks could be assigned by comparison with the HPAE-PAD mapping database—with a deviation of $<\pm 0.5\%$ in 8 cases and $<\pm 0.7\%$ in 1 case (peak 3). The assigned *N*-glycan structures are listed in Table 4.

Structural Assignment of the Asialo N-Glycans of rhuEPO (BHK)

The asialo *N*-glycan pool of rhuEPO (BHK), obtained upon desialylation of the PNGase F-derived *N*-glycans, as measured by gradient A, with Neu5Ac and Neu5Gc as internal standards, is shown in Fig. 4. Six of the eight detected structures (peaks 2–8) could be assigned (Table 5) by comparison with the mapping database, after the measured RTs were corrected against the RTs of the internal standards (peaks 1 and 10).

As can be seen in Table 5, the tetraantennary structures C4-046301, C4-057301.1R-A2M6, and C4-068301.1R-A2M6.1R-A6M6 were deduced from the database with a deviation of $<\pm 0.1\%$. The two triantennary isomers C3-035301.A6M6 and C3-035301.A4M3 were found with a deviation of $<\pm 0.5\%$ and $<\pm 0.7\%$, respectively, and the biantennary structure C2-024301 was detected with a deviation of $<\pm 1\%$, which could be due to the asymmetry of the peak.

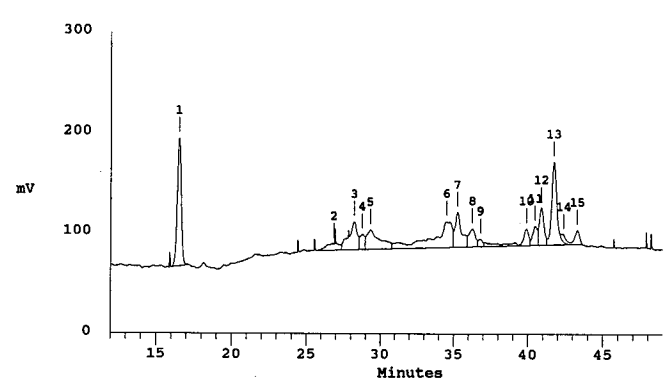


FIG. 3. HPAE-PAD chromatogram of the *N*-glycans of rhuEPO (BHK), measured with gradient S, using Neu5Ac as internal standard.

TABLE 4

Analysis of the *N*-Clycans of rhuEPO (BHK) by Comparison with Structures Contained in the Mapping Database, Using Gradient *S*

Peak no.	Identified structure	Deviation (%)
15	Not identified	—
14	Not identified	—
13	C4-446301.40	<0.2
12	C4-457301.40.1R-A2M6	<0.5
11	C4-457301.40.1R-A6M6	<0.5
10	C4-468301.40.1R-A2M6.1R-A6M6	<0.5
8	C3-335301.30.A4M3	<0.5
	C3-335301.30.A6M6	<0.5
7	C4-346301.30 Isomer 1	<0.5
6	C4-357301.30.1R	<0.5
5	C2-224301.20	<0.5
3	C4-246301.20	<0.7
	C4-257301.20.1R	<0.7
2	Not identified	—
1	Internal Neu5Ac	—

TABLE 5

Analysis of the Asialo *N*-Glycans of rhuEPO (BHK) by Comparison with Structures Contained in the Mapping Database, Using Gradient *A*

Peak No.	Identified structure	Deviation (%)
10	Internal Neu5Gc	—
9	Not identified	—
8	C4-068301.1R-A2M6.1R-A6M6	<0.01
7	C4-057301.1R-A2M6	<0.01
6	Not identified	—
5	C4-046301	<0.01
4	C3-035301.A6M6	<0.05
3	C3-035301.A4M3	<0.07
2	C2-024301	<0.10
1	Internal Neu5Ac	—

DISCUSSION

We have evaluated and validated the suitability of HPAEC for *N*-glycan analysis in order to establish a carbohydrate mapping database, destined for the carbohydrate structural assignment by mere composition of retention times. Such an idea actually goes back to the great pioneering work of Kobata and colleagues, who were able to assign the structures of neutral carbohydrates on the basis of the effective size of the sugar, expessed in glucose units (16,17). Unfortunately, Kobata's Bio-Gel P-4 (<400 mesh) column chromatography separation is a very time-consuming process, which is restricted to neutral carbohydrates and relies on the introduction of a tritium label.

Tomiya *et al.* (2), in their establishment of a 2D carbohydrate mapping database, used ODS–silica and amide–silica columns for separation in two dimensions and employed PA-glucose oligomers as external standard. Thus, they were able to express the retention times of their PA-oligosaccharides as defined glucose unit numbers (by comparing the elution position of each of their PA-oligosaccharides with those of the standard PA-glucose oligomers). The values of the PA-glucose unit numbers reported were reproducible within an experimental error of 3%.

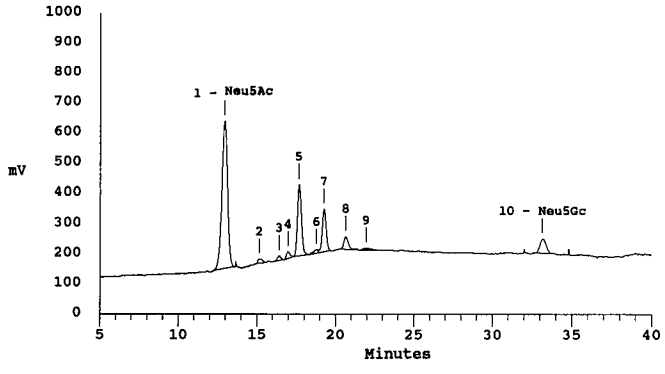


FIG. 4. HPAE-PAD chromatogram of the asialo *N*-glycans of rhuEPO (BHK), measured with gradient A, using Neu5Ac and Neu5Gc as internal standards.

Although this carbohydrate mapping database has found fruitful application in the structural analysis of carbohydrate chains (18–20), the analytical procedure has the disadvantage of requiring multistep derivatization and clean-up processes. Another major drawback of the procedure is its limitation to asialo structures, thus not answering the very important question of the sialylation status of the glycans (number of Neu5Ac residues and linkage positions). However, knowledge about the sialylation status is particularly important with certain recombinant glycoproteins, such as erythropoietin and antithrombin III, where the presence of sialic acid is required for biological activity, as desialylated structures are rapidly cleared by interaction with asialo receptors in the liver.

HPAE-PAD, on the other hand, allows oligosaccharide separation and detection without requiring prior derivatization and appears therefore superior to the existing PA-mapping technique.

To our knowledge, Spellman *et al.* (21) were the first to make use of HPAEC as an integral part of the carbohydrate structure elucidation of a novel molecule, analyzing the carbohydrate structures of recombinant *t*-PA, expressed in Chinese hamster ovary cells. Here we present the systematic continuation, improvement, and generalization of this strategy.

Honda *et al.* (22) have reported that, in their HPAEC experiments with high-mannose-type structures and di- and triantennary sialylated alditols, isocratic separa-

tions provided a relative standard deviation of less than 0.1%, while gradient elution increased the CV to about 5%. (Following the nomenclature proposed in this paper, Honda's structures read C2-124301.01.S6-A2M3.ol, C2-134301.01.S6-A2M3.G3-A2M6.ol, C2-22-4301.02.ol, and C3-235301.11.A4M6.S3-A4M6.ol).

We have now developed two linear gradients S and A that allow optimal separation of sialylated (S) and asialo (A) *N*-glycans on a CarboPac PA-100 column (Table 1). Moreover, we have found that the introduction of Neu5Ac as internal standard for *N*-glycan measurements by HPAE-PAD reduces the degree of variation of the retention times to less than 0.5%, which significantly increases the accuracy of the measurements (Table 2) and, as a consequence, the reliability of the database. A coefficient of variation of the retention times at multiple runs on different days of <0.5% was defined as the prerequisite for reliable measurements. The most accurate and most reliable retention times of *N*-glycans were observed using two internal standards per measurement, i.e., Neu5Ac and (Neu5Ac)3 (in gradient S) and Neu5Ac and Neu5Gc (in gradient A), respectively, and with the measured retention times of the glycans corrected accordingly.

Both methods S and A were extensively validated, using two different Dionex Bio-LC systems (Hermentin *et al.*, unpublished results). Only one of the two Bio-LC systems actually fulfilled our dogmatic requirement of allowing multiple measurements at different days with a degree of variation of less than 0.5%, and this system was finally used to establish the *N*-glycan mapping database.

In order to enable proper search profiles in the Lotus 1–2–3 database, the existing nomenclature or numbering of the *N*-glycans had to be improved and standardized, as explained in more detail under Experimental and illustrated in Scheme 1. Although this type of numbering is far from completion, it covered satisfactorily all the *N*-glycans thus far present in the database and allowed proper search profiles and the comparison of retention times of distinct structures.

For better schematic presentation of the database, the retention times of the structures measured in HPAEC (*x*-axis) were plotted against their corresponding number of monosaccharide residues (*y*-axis), and extracts of 48 and 15 *N*-glycans of the database are presented in Figs. 1 and 2, respectively.

On the basis of the approximately 100 *N*-glycan structures that have thus far entered the database, valuable information could be gained with respect to structural alterations:

The introduction of one, two, or three LacNAc repeats into asialo structures, such as C4-046301 (Fig. 2, upward arrows), resulted in increased retention times, whereas the introduction of LacNAc repeats into sialylated structures decreased the RTs. These latter results are not in line with the presumed general trend in HPAEC, according to which oligosaccharides of a homologous series elute in the order of increasing size (23–25), and further exceptions from the presumed rule have been provided by others (25).

The shifts in retention time observed upon defucosylation (Fig. 2, downward arrows), the introduction of Gal α1,3 epitopes, and β-galactosidase treatment, as well as the elution order of the triantennary asialo isomers ($\cdots$A4M3 < $\cdots$A6M6), of the Neu5Ac linkage position ($\cdots$Neu5Ac α2,6 < $\cdots$Neu5Ac α2,3), and of the antennarity (C2– < C3– < C4–) were in line with various reports in the literature (for reviews see (26,27)).

In order to verify the suitability and reliability of the database for the structural determination of *N*-glycans by mere comparison of retention times, the sialylated and desialylated *N*-glycan pool of a rhuEPO (BHK) preparation (5) was measured by HPAE-PAD, using the optimized linear gradients S and A, respectively. Nine peaks of the sialylated *N*-glycan pool (Fig. 3) could be assigned by comparison with the mapping database (Table 4), with a deviation in the range of only ±0.5%, and six of the eight peaks of the asialo *N*-glycans detected by HPAE-PAD (Fig. 4) could be assigned with a degree of variation of <1%, three of them even with a deviation of <±0.1% (Table 5). It is noteworthy that this *N*-glycan analysis of rhuEPO (BHK) was performed approx. 6 months after the database was established, at a time when the analytical laboratory had moved to a different building. Thus, we are convinced that the corrected retention times of the established database are appropriate and reliable numbers, even for work in different laboratories. As only less than 100 pmol of glycan is necessary for accurate and reliable measurements by HPAE-PAD, the analysis of glycoproteins, such as rhuEPO, requires only about 100 μg of glycoprotein for the analysis of both the sialylated and the asialo structures, using gradients S and A, respectively. As neither derivatization nor the introduction of a radiolabel is required for these measurements, mapping by HPAE-PAD appears to be the most powerful and quickest method for carbohydrate structural assignment yet available.

In parallel, we have developed a similarly powerful method for the separation of *N*-glycans by capillary electrophoresis, which may be used as an appropriate second dimension for the desired 2D mapping of *N*-glycans (28).

It is expected that the mapping strategy presented here, in connection with exo- and endoglycosidase treatment, will enable quick and facile structural elucidation of *N*-glycans by mere comparison of retention times and thus reduce the high-cost and time-consuming demands of carbohydrate structural analysis.

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