ORIGINAL RESEARCH

Increased branching and sialylation of N-linked glycans correlate with an improved pharmacokinetic profile for BAY 81–8973 compared with other full-length rFVIII products

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Background: BAY 81–8973 (Kovaltry) is an unmodified full-length recombinant factor VIII (rFVIII) for treatment of hemophilia A. The BAY 81–8973 manufacturing process results in a product of enhanced purity with a consistently high degree of branching and sialylation of N-linked glycans. This study evaluated whether a relationship exists between N-linked glycosylation patterns of BAY 81–8973 and two other rFVIII (sucrose-formulated rFVIII [rFVIII-FS; Kogenate FS]) and antihemophilic factor (recombinant) plasma/albumin-free method (rAHF-PFM; Advate) and their pharmacokinetic (PK) characteristics.

Materials and methods: N-linked glycans or terminal carbohydrates were enzymatically removed from immobilized BAY 81–8973, rFVIII-FS, and rAHF-PFM proteins and analyzed using high-performance liquid chromatography to determine the percentage of individual N-linked glycan structures and degree of sialylation of each structure. PK data were available from two separate phase 1 crossover studies in which the PK profile of BAY 81–8973 was compared with that of rFVIII-FS (n=26) and rAHF-PFM (n=18) in patients with severe hemophilia A who received a single 50 IU/kg dose of each product.

Results: BAY 81–8973 and rFVIII-FS had increased N-linked glycan branching with higher levels of sialylation compared with rAHF-PFM. Levels of trisialylated glycans were 29.0% for BAY 81–8973 vs 11.5% for rFVIII-FS and 4.8%–5.5% for rAHF-PFM; tetrasialylated glycans were 12.0% vs 2.8% and 0.6%, respectively. Degree of sialylation was 96% for BAY 81–8973, 94% for rFVIII-FS, and 78%–81% for rAHF-PFM. Based on chromogenic assay results from the single-dose phase 1 PK studies, BAY 81–8973 half-life was 15% longer than that for rFVIII-FS and 16% longer than rAHF-PFM.

Conclusion: Increased N-glycan branching and sialylation were seen for BAY 81–8973 vs rFVIII-FS and rAHF-PFM. Improved PK for BAY 81–8973 relative to rFVIII-FS and rAHF-PFM as seen in single-dose crossover PK studies might be related to this greater level of branching and sialylation, which can prolong the time BAY 81–8973 remains in the circulation.

Keywords: clearance, glycan structure, glycosylation, half-life

Introduction

Hemophilia A is a congenital bleeding disorder resulting from insufficient levels of clotting factor VIII (FVIII).[1] Patients with severe hemophilia A have FVIII levels <1% of normal, putting them at risk for spontaneous bleeding episodes into tissues, muscles, and joints; repeated bleeding into joints can lead to chronic hemophilic arthropathy. The current standard of care for hemophilia A is prophylactic infusions of a FVIII product.

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For standard-acting FVIII products, infusions are typically administered every other day or three times per week, given the ~12-hour half-life of FVIII,[2,3] with frequent infusions presenting a considerable burden for patients and their caregivers.[3]

The circulatory half-life of recombinant FVIII (rFVIII) products is affected by glycosylation of the FVIII protein, including N-linked glycan branching and terminal sialic acid occupancy, primarily through receptor-mediated hepatic clearance (eg, asialoglycoprotein receptor [ASGPR] and lipoprotein receptor-related protein [LRP]).[4,5] Through glycosylation, a post-translational modification, carbohydrate structures are covalently attached to a protein backbone;[6] the most common structures are N-linked and O-linked glycans.[7] The glycosylation process begins during translocation of the protein into the endoplasmic reticulum, with further modification of the carbohydrate structures on the nascent protein in the Golgi.[6] These protein-bound carbohydrates are important for the structure and function of the FVIII protein, contributing to optimal protein folding and binding to ASGPR via N-linked glycans in the FVIII B domain, which plays a role in protein clearance;[4,6] glycans not capped with sialic acid are rapidly cleared.[6,8,9]

BAY 81–8973 (Kovaltry, Bayer, Berkeley, CA, USA) is an unmodified, full-length rFVIII approved for prophylaxis and on-demand treatment of bleeding episodes in patients with hemophilia A.[10] BAY 81–8973 is translated as a single-chain 330-kD precursor with a domain structure of A1-A2-B-A3-C1-C2 subunits.[10] Post-translational proteolytic processing yields an A1-A2-B heavy chain and an A3-C1-C2 light chain to form a large heterodimeric structure linked by a divalent cation bridge.[10] BAY 81–8973 is highly glycosylated, with multiple N-linked and O-linked glycans present on the structure, predominantly within the B domain, as well as six highly occupied tyrosine sulfation sites.[10]

The BAY 81–8973 manufacturing process is based on that used for sucrose-formulated rFVIII (rFVIII-FS; Kogenate FS, Bayer), with changes and enhancements made to improve production efficiency, further augment pathogen safety, and eliminate animal- and human-derived raw materials from the production processes.[10] Both rFVIII-FS and BAY 81–8973 are full-length recombinant human FVIII products expressed in baby hamster kidney (BHK21) cells. The BHK21 cell line used for BAY 81–8973 was developed by introducing the gene for human heat shock protein 70 (HSP70) into the cell line used to manufacture rFVIII-FS (a full-length rFVIII expressed in BHK cells in the presence of human purified plasma); this change improved cell line robustness

and productivity.[10] Co-expression of HSP70 along with the FVIII gene in the production cell line is likely responsible for improving stable and reproducible FVIII expression by inhibiting apoptosis during cell culture, thus keeping FVIII producing cell viability higher. It is believed that HSP70 facilitates appropriate protein folding of the nascent intracellular rFVIII polypeptides.[11,12] The chaperone function of HSP70 co-expressed with rFVIII may result in larger antennary glycan structures and a higher percentage of sialylation by reducing misfolded proteins during transport through the endoplasmic reticulum and golgi where N-linked glycosylation occurs intracellularly.[13] Overall, the BAY 81–8973 manufacturing process results in a stable product with a consistently high degree of sialylation of N-linked glycans on the molecular surface, as well as enhanced purity, with low levels of high-molecular-weight impurities and protein aggregates.[10]

Given that rFVIII products have different patterns of glycosylation and sialylation, our objective was to evaluate the correlation between the N-linked glycosylation of BAY 81–8973, rFVIII-FS, and antihemophilic factor (recombinant) plasma/albumin-free method (rAHF-PFM; Advate, Shire, Westlake Village, CA, USA) and their pharmacokinetic (PK) profile.

Materials and methods
Glycan analysis
The N-linked glycans or terminal carbohydrates were enzymatically removed from immobilized BAY 81–8973, rFVIII-FS, and rAHF-PFM, and analyzed using high-performance liquid chromatography (HPLC) to determine the percentage of individual N-linked glycan structures (neutral, mono-, di-, tri-, and tetra-antennary glycans) and the degree of sialylation of these N-glycans. Specifically, the oligosaccharide map method was used to provide a quantitative chromatographic profile of all N-linked glycan structures. Using this method, N-linked oligosaccharides were released from the rFVIII samples and bound to nylon spin filters using PNGase F (Prozyme/Glyko, Hayward, CA, USA). The released N-glycans were then labeled with anthranilic acid (2-AA; Spectrum Chemicals, New Brunswick, NJ, USA) using reductive amination with sodium cyanoborohydride (reagent grade) as the reductant. The 2-AA labeled components of the rFVIII preparations were separated into peaks using HPLC (Agilent, Santa Clara, CA, USA) in normal phase/ion exchange mixed mode (Shodex Asahi Pak NH2P-50 2D, Showa Denko America, Inc., New York, NY, USA) and using fluorescence emission at 425 nm after
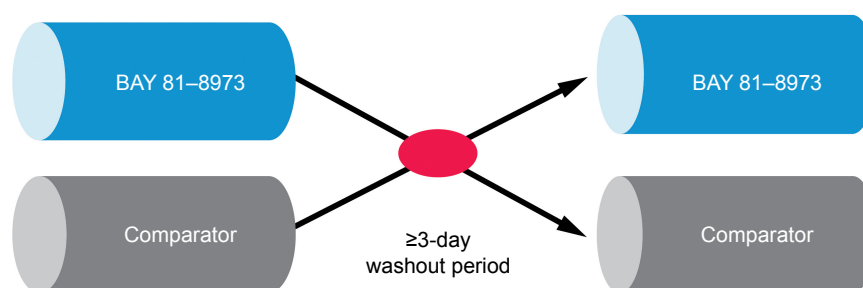
Figure I Phase I crossover study design for pharmacokinetic comparison of BAY 81–8973 vs rFVIII-FS and rAHF-PFM.
Note: rFVIII-FS and rAHF-PFM were used as comparators in separate phase I studies.
Abbreviations: rAHF-PFM, antihemophilic factor (recombinant) plasma/albumin-free method; rFVIII-FS, sucrose-formulated recombinant factor VIII.

excitation at 360 nm. This procedure separates the labeled components primarily according to their ionic charge, which is dependent on the number of sialic acid groups covalently bound to the oligosaccharides, and also by their size and structure.[14,15]

The sialylation and alpha-galactose method was used to quantify the glycosylation of each rFVIII product with respect to termination of branching of the N-linked glycan structures. This method involves binding the rFVIII samples to a nylon membrane, followed by the enzymatic release of the exposed α-linked galactose residues using α-galactosidase (Prozyme/Glyko). This step is followed by enzymatic release of the exposed β-linked galactose residues (uncapped) using β-galactosidase (Prozyme/Glyko). The "capped" β-linked galactose residues are then released using a combination of β-galactosidase and sialidase A (Prozyme/Glyko). Each digest was then analyzed using high pH anion-exchange chromatography, coupled with a pulsed amperiometric detector (HPAEC-PAD; Dionex, Sunnyvale, CA, USA), to measure the relative amount of galactose released during the respective digests. Because β-galactosidase is a glycoprotein, blank digestions (with no rFVIII) were run for both digests, the β-galactosidase and the sialidase A+β-galactosidase sample preparations, and subtracted from the peak area obtained in those digests. By calculating specific ratios for these adjusted peak areas, values for the percentage of α-galactose and the percentage of sialic acid capping were determined.

Pharmacokinetic comparison

The PK of BAY 81–8973 was compared with that of rFVIII-FS and rAHF-PFM in two separate randomized phase 1 crossover studies (study design is shown in Figure 1).[16,17] Each study enrolled patients aged 12–65 years (rFVIII-FS comparison) or 18–65 years (rAHF-PFM comparison) with severe hemophilia A ≥150 exposure days to any FVIII product, and no history of FVIII inhibitors.[16,17] All patients

received a single 50 IU/kg infusion of BAY 81–8973 or the comparator product, followed by crossover to a single infusion of the other product after a ≥3-day washout period (Figure 1).[16,17] In both studies, plasma samples were collected predose and 0.25, 0.5, 1, 3, 6, 8, 24, 30, and 48 hours postdose for determination of PK parameters using one-stage and chromogenic assays.[16,17]

Results

Glycan analysis

N-glycan analysis of different batches of BAY 81–8973 (n=65), rFVIII-FS (n=3), and rAHF-PFM (n=6) indicated that BAY 81–8973 and rFVIII-FS had higher levels of sialylation and increased N-linked glycan branching compared with rAHF-PFM (Figures 2 and 3). Levels of trisialylated glycans were 29.0% for BAY 81–8973, 11.5% for rFVIII-FS, and 4.8%–5.5% for rAHF-PFM. For tetrasialylated glycans, levels were 12.0%, 2.8%, and 0.5%–0.6%, respectively. The degree of sialylation was higher for BAY 81–8973 (96%) compared with rFVIII-FS (94%) and rAHF-PFM (78%–81%).

Pharmacokinetic comparison

Twenty-six patients (median age=29.5 years) completed the BAY 81–8973 vs rFVIII-FS PK study, and 18 patients (median age=36.0 years) completed the BAY 81–8973 vs rAHF-PFM PK study.[16,17] In both studies, BAY 81–8973 displayed an improved PK profile vs the comparator product (Table 1).[16,17]

The half-life of BAY 81–8973 was 10% and 15% longer than that for rFVIII-FS (P=0.01 and P=0.002), based on the one-stage assay and chromogenic assay, respectively, and the area under the curve (AUC) was 19% higher for BAY 81–8973 vs rFVIII-FS, based on both assays (P<0.0001 for one-stage assay; P=0.0003 for chromogenic assay).[16] Compared with rAHF-PFM, BAY 81–8973 had a half-life that was 24% longer based on the one-stage assay and 16%
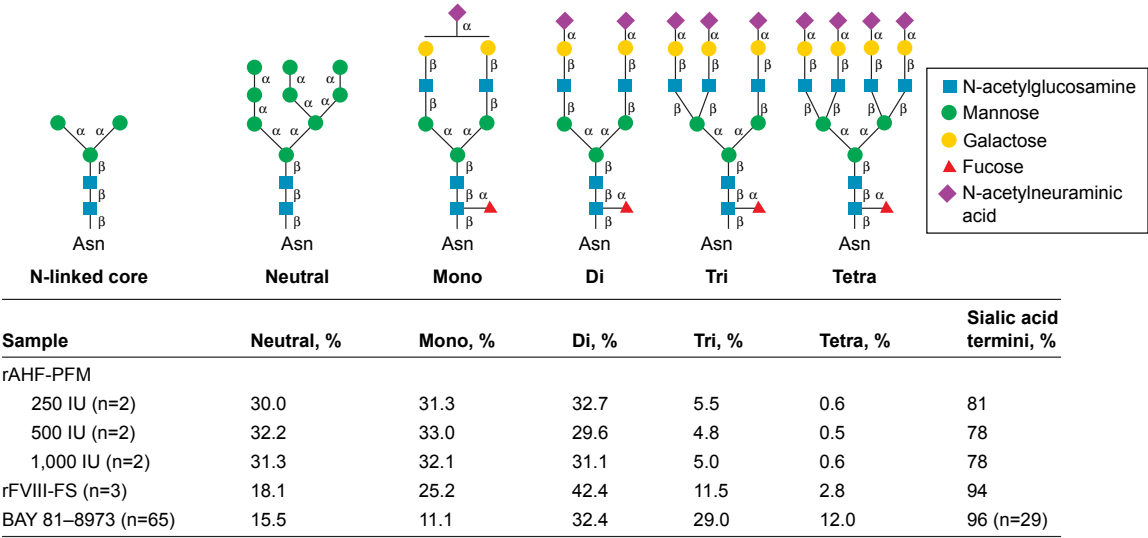
Figure 2 Glycan structures in BAY 81–8973, rFVIII-FS, and rAHF-PFM.
Note: Glycan structures drawn using DrawGlycan-SNFG.[22]
Abbreviations: rAHF-PFM, antihemophilic factor (recombinant) plasma/albumin-free method; rFVIII-FS, sucrose-formulated recombinant factor VII.
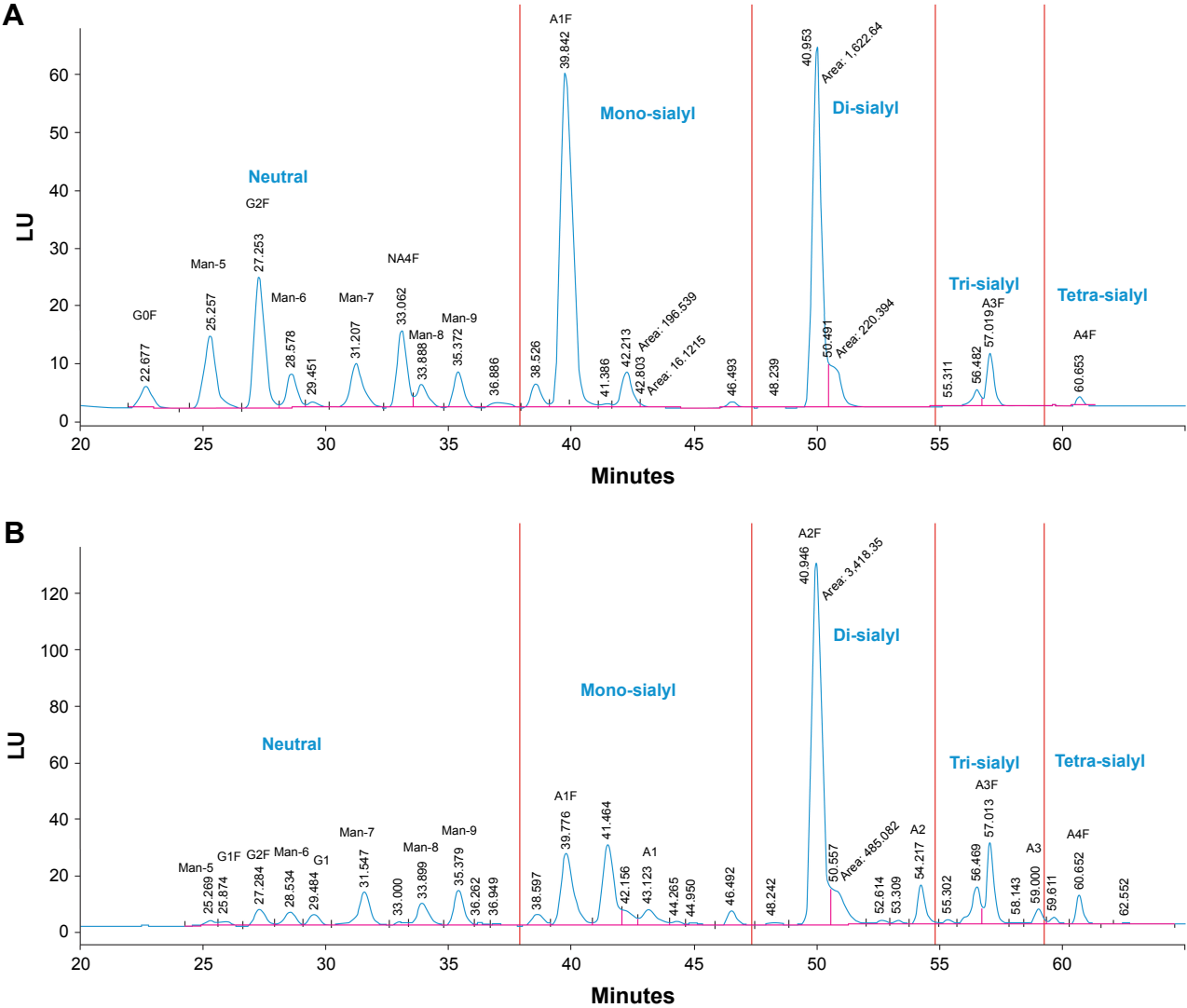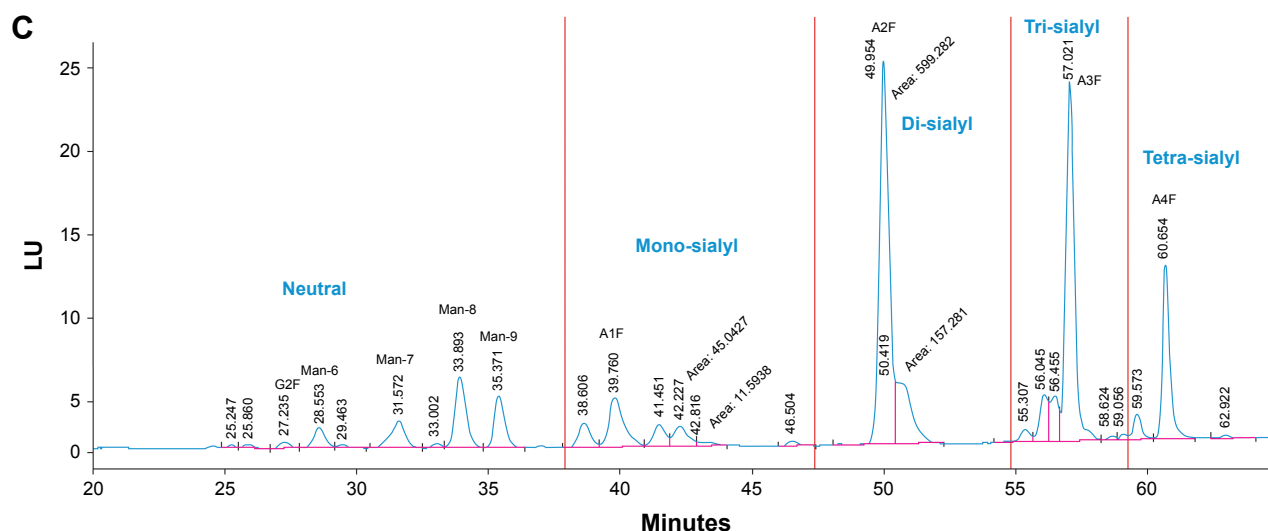
Sample	Neutral, %	Mono, %	Di, %	Tri, %	Tetra, %	Sialic acid termini, %
rAHF-PFM						
250 IU (n=2)	30.0	31.3	32.7	5.5	0.6	81
500 IU (n=2)	32.2	33.0	29.6	4.8	0.5	78
1,000 IU (n=2)	31.3	32.1	31.1	5.0	0.6	78
rFVIII-FS (n=3)	18.1	25.2	42.4	11.5	2.8	94
BAY 81–8973 (n=65)	15.5	11.1	32.4	29.0	12.0	96 (n=29)

Figure 3 (Continued)

Figure 3 Chromatograms of the N-linked glycan charge separations for (**A**) rAHF-PFM; (**B**) rFVIII-FS; and (**C**) BAY 81–8973.
Notes: Separation of the neutral, mono-, di-, tri-, and tetra-sialylated N glycans are shown in their respective retention time windows. Major glycan structures are annotated.
Abbreviations: LU, luminescence units; rAHF-PFM, antihemophilic factor (recombinant) plasma/albumin-free method; rFVIII-FS, sucrose-formulated recombinant factor VIII.

longer based on the chromogenic assay ($P<0.0001$ for both assays); AUC from time 0 to infinity was 32% and 48% higher, respectively, for BAY 81–8973 vs rAHF-PFM with both assays ($P<0.0001$).[17] Clearance was significantly slower for BAY 81–8973 vs rFVIII-FS and rAHF-PFM with both assays ($P<0.0001$).[16,17] The FVIII concentration-time profiles showed that, at most time points, FVIII levels were higher for BAY 81–8973 compared with rFVIII-FS and rAHF-PFM (Figure 4).[17,23]

Correlation of glycan branching and sialylation with half-life

Increases in the percentage of sialylated tri-antennary and tetra-antennary N-glycans correlated well with longer half-life (adjusted $R^2=0.978$ and 0.892 for tri-antennary and

tetra-antennary N-glycans, respectively; Figure 5). Higher percentages of sialylation (ie, sialic acid capping) correlated with a longer half-life (adjusted $R^2=0.697$), but the relationship was not as strong as that for glycan branching (Figure 5).

Discussion

Differences were seen in N-glycans among the three rFVIII products studied, with greater glycan branching and sialylation seen with BAY 81–8973 compared with rFVIII-FS and rAHF-PFM. This observation is consistent with a previously published report by Baunsgaard et al.[18] In addition, in published PK studies, BAY 81–8973 showed an improved PK profile (longer half-life, higher AUC, slower clearance) vs the two comparator products.[16,17]

Table 1 Pharmacokinetic results after single 50 IU/kg dose administration of BAY 81–8973, rFVIII-FS, and rAHF-PFM using the chromogenic assay

	BAY 81–8973 vs rFVIII-FS (N=26)			BAY 81–8973 vs rAHF-PFM (N=18)		
	BAY 81–8973	**rFVIII-FS**	**P-value**	**BAY 81–8973**	**rAHF-PFM**	**P-value**
AUC_{0-inf}, IU·h/dL	1,889.2 (36.1)	1,583.9 (39.9)	0.0003	2,440 (28.5)	1,650 (31.0)	<0.0001
C_{max}, IU/dL	130.1 (23.0)	136.2 (23.8)	0.45	151 (19.9)	153 (17.1)	0.32
MRT, h	19.3 (26.8)	16.5 (27.4)	<0.0001	19.2 (27.4)	15.0 (27.9)	<0.0001
CL, dL/h/kg	0.026 (36.1)	0.032 (39.9)	0.0003	0.021 (28.5)	0.030 (31.0)	<0.0001
Half-life, h	13.8 (28.0)	12.0 (28.2)	0.002	13.9 (25.1)	12.0 (23.3)	<0.0001
Range	7.7–23.7	6.4–20.5		9.95–22.2	9.06–17.9	
Arithmetic mean	14.3	12.4		NA	NA	
V_{ss}, IU/dL	0.51 (31.0)	0.52 (32.0)	0.67	0.39 (19.1)	0.46 (16.7)	<0.0001

Notes: Data are geometric mean (% coefficient of variation) unless otherwise indicated. Copyright ©2015. John Wiley & Sons Ltd. Adapted from Shah A, Delesen H, Garger S, Lalezari S. Pharmacokinetic properties of Bay 81-8973, a full-length recombinant factor VIII. *Haemophilia*. 2015;21(6):766–771. © 2015 John Wiley & Sons Ltd.[16] Adapted by permission from Springer Nature under the terms of the Creative Commons Attribution-NonCommercial 4.0 International License (http://creativecommons.org/licenses/by-nc/4.0/): Springer Nature:*Clin Pharmacokinet*. Improved pharmacokinetics with Bay 81-8973 versus antihemophilic factor (recombinant) plasma/ albumin-free method: a randomized pharmacokinetic study in patients with severe hemophilia A. Shah A, Solms A, Garmann D, et al. 2017;56(9):1045–1055.[17]
Abbreviations: AUC_{0-inf}, area under the curve from time 0 to infinity; CL, clearance; C_{max}, maximum concentration; FVIII, factor VIII; MRT, mean residence time; NA, not available; V_{ss}, volume of distribution at steady state; rAHF-PFM, antihemophilic factor (recombinant) plasma/albumin-free method; rFVIII-FS, sucrose-formulated recombinant factor VIII.
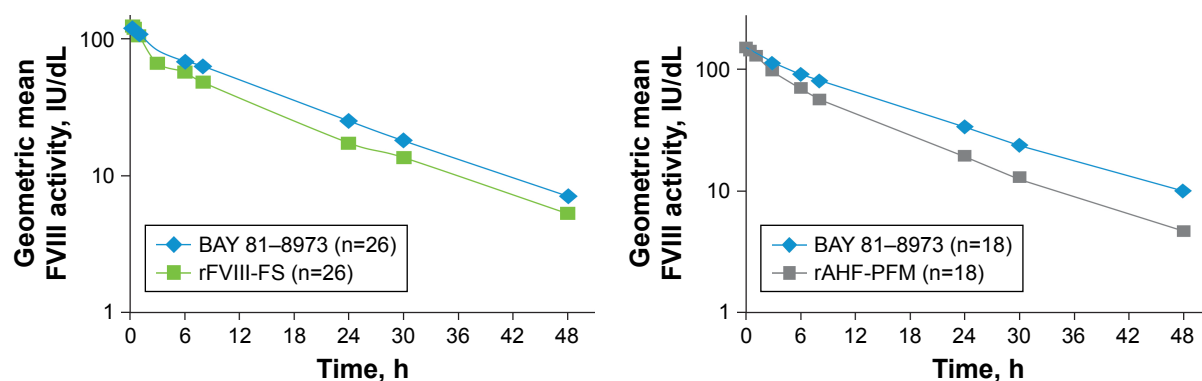
Figure 4 FVIII concentration-time curves after single 50 IU/kg dose administration of BAY 81–8973, rFVIII-FS, and rAHF-PFM using the chromogenic assay.[17,23]
Note: Reprinted by permission from Springer Nature: Springer Nature:*Clin Pharmacokinet.* Improved pharmacokinetics with Bay 81-8973 versus antihemophilic factor (recombinant) plasma/ albumin-free method: a randomized pharmacokinetic study in patients with severe hemophilia A. Shah A, Solms A, Garmann D, et al. 2017;56(9):1045–1055.[17] This poster was originally published in *Blood* 132:1209. Teare et al. Improved Pharmacokinetic Profile for BAY 81-8973. Due to Increased Branching and Sialylation of N-Linked Glycans of Recombinant Factor VIII. Poster presented at: American Society of Hematology; December 1–4; 2018; San Diago, USA. © the American Society of Hematology.[23]
Abbreviations: FVIII, factor VIII; rAHF-PFM, antihemophilic factor (recombinant) plasma/albumin-free method; rFVIII-FS, sucrose-formulated recombinant factor VIII.

Improved PK for BAY 81–8973 vs rAHF-PFM was also seen in simulations based on population PK (popPK) models. In a popPK model developed using chromogenic assay data from the phase 1 BAY 81–8973 vs rAHF-PFM study,[17] median time to reach a FVIII level of 1 IU/dL was estimated to be ~18 hours longer for BAY 81–8973 vs rAHF-PFM (80.5 vs 62.5 hours) after a 25 IU/kg infusion. In a popPK analysis based on data extracted from the Web-Accessible Population Pharmacokinetic Service–Hemophilia (WAPPS-Hemo) database, the estimated terminal half-life
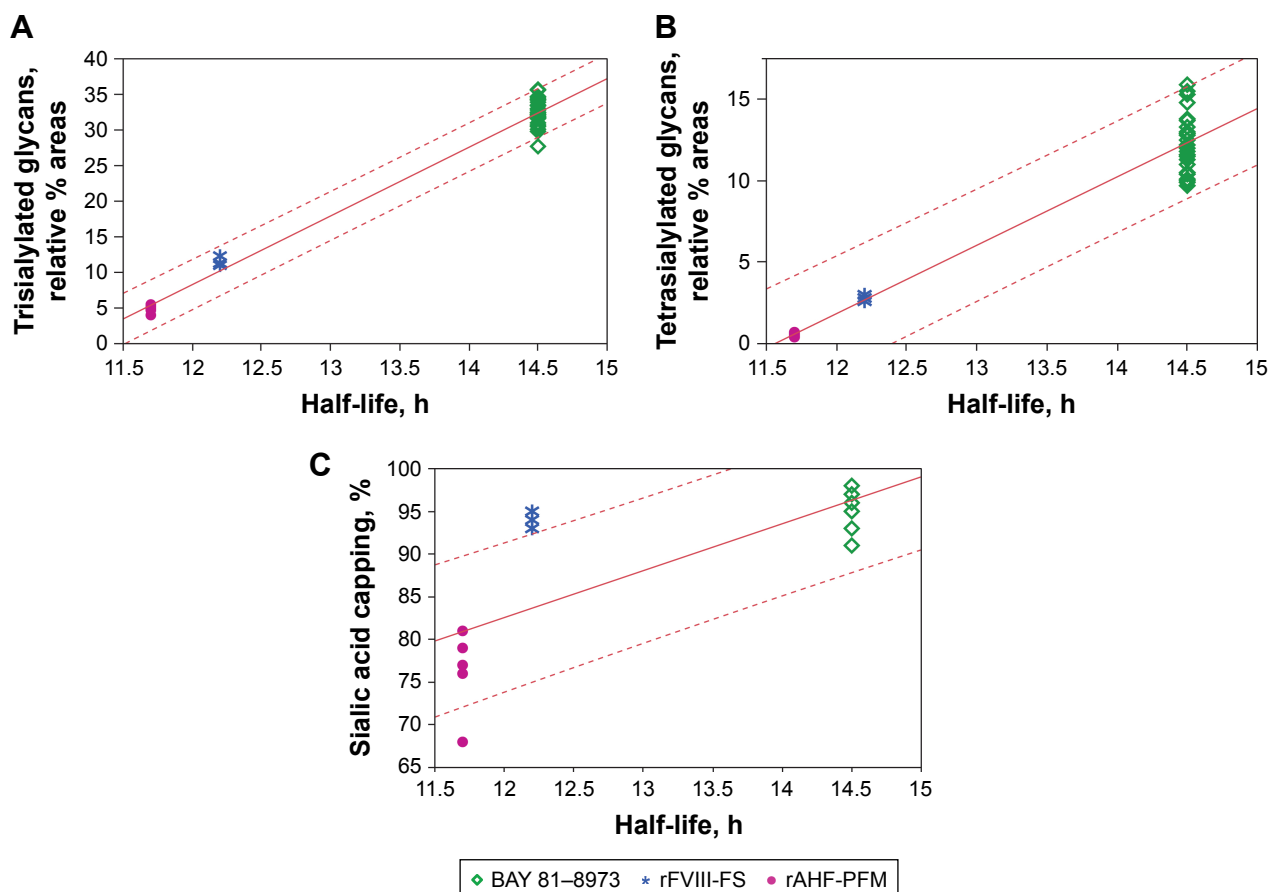

Figure 5 Correlation of BAY 81–8973, rFVIII-FS, and rAHF-PFM half-life with percentage of (**A**) trisialylated glycan branching, (**B**) tetrasialylated glycan branching, and (**C**) sialic acid capping.
Note: Solid line is the linear regression fit; dashed line is the 95% CI.
Abbreviations: rAHF-PFM, antihemophilic factor (recombinant) plasma/albumin-free method; rFVIII-FS, sucrose-formulated recombinant factor VIII.

for BAY 81–8973 was 13.1 hours, compared with 10.5 hours for rAHF-PFM.[19]

The circulatory half-life of rFVIII products is affected by glycosylation of the FVIII protein, including N-linked glycan branching and terminal sialic acid occupancy, primarily through receptor-mediated hepatic clearance (eg, ASGPR and LRP).[4,5] The longer circulation time and longer half-life for BAY 81–8973 compared with rFVIII-FS and rAHF-PFM might be related to the increased branching and sialylation of the N-glycans in BAY 81–8973. This increased glycan branching and sialylation may shield BAY 81–8973 from clearance by known hepatocyte receptors, such as ASGPR, which bind to exposed galactose on the glycan structure, as well as LRP. Similar observations have been made with other clotting factors such as recombinant activated factor VII and recombinant factor IX.[20,21]

Conclusion

The differences seen in N-glycan structure and degree of sialylation of BAY 81–8973, rFVIII-FS, and rAHF-PFM may influence their PK profile, with BAY 81–8973 displaying improved PK compared with the comparator products; increased glycan branching and sialylation correlated well with increased half-life. However, further study is needed to determine the exact mechanisms underlying the relationship between glycan branching, sialylation, and prolonged FVIII circulation.

Acknowledgments

Disclosure

John M Teare, David S Kates, Anita Shah, and Stephen Garger are employees of Bayer. The authors report no other conflicts of interest in this work.

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