Ⓑ 製　剤

製剤の品質特性を考える際には，機能的な面を含め製剤そのものの品質評価およびその構成成分である原薬や添加剤の品質を十分に評価する．多くの製剤に共通して評価する項目と剤形の機能に関連し，剤形ごとに評価すべき項目がある．

1．添加剤の評価

賦形剤，結合剤，崩壊剤，滑沢剤，pH調節剤，酸化防止剤，膜透過性促進剤，放出制御剤など各種の添加剤が原薬に追加されて製剤が完成する．添加剤に関して，それらが目的とする機能性を発揮し，かつ製剤の有効期間を通じて役割を果たしうる能力を評価することが必要である．例えば，原薬と添加剤，あるいは添加剤を2成分組み合わせて使用する場合など，原薬と添加剤あるいは添加剤と他の添加剤の配合特性についても評価しておく必要がある．

添加剤の品質の微妙な変化が製剤機能の大きな変化につながることがある．滑沢剤のステアリン酸マグネシウムが，BSEのリスクを回避するためにウシ由来のものから植物性に切り替えられたときに，滑沢剤の粒子が細かくなって表面分布状態が変化，溶出性が低下し，製薬会社は対応に追われたことがある．

2．共通して評価する項目

共通して評価する項目としては，製剤中の原薬の確認，含量(力価)および純度の評価が含まれる．

製剤中の原薬の評価法を確立しておくことは重要である．製剤化のプロセスは原薬に添加剤が加えられ，製剤機能が付加される過程であり，規定どおりの原薬が用いられていれば，製剤中に原薬が存在していることは自明のはずである．しかし，GMPの管理を実施してもリスクは残り，最終製品で再確認をする必要がある．さらに現在では，原薬と製剤の工場が異なるのは無論のこと，素錠までの工程とコーティング工程が異なる，あるいは錠剤バルク工程とパッケージング工程で工場が異なることは珍しくない．複雑化したサプライチェーンに起因する事故を防ぐためにも，最終製品で原薬の存在を確認することの重要性は増している．

製剤中の原薬の確認は，添加剤中から原薬を同定する必要があるため，添加剤の妨害がなく，かつ存在すると考えられる類似構造をもつ化合物と識別できる方法である必要がある．HPLCやTLCを組み合わせて評価されることが多い．

含量の評価(定量)も，基本的に考慮すべき事項は医薬品原薬における定量と同じであるが，添加剤の妨害を受けない評価法である必要があり，HPLCによる定量が実施されることが多い．

不純物の評価に関しては，製剤化のプロセスおよび製剤の保存中における分解，あるいは添加剤と反応して生成する不純物を特定し，許容される基準を設定する必要がある．原薬の合成工程に由来する反応中間体などの不純物に関しては，原薬で管理されているので，改めて製剤で許容基準を設定する必要はない．

表2-3 ● 剤形に応じて必要となる製剤試験

剤形	評価すべき項目*1
経口固形製剤	溶出性，崩壊性，硬度／摩損度，投与単位の均一性，水分含量，微生物限度
経口液剤	投与単位の均一性，pH，微生物限度，抗菌性保存剤含量，溶出物，アルコール含量，溶出性，粒子径分布(粒度)，再分散性，流動学的性質，再調製時間，水分含量
注射剤	投与単位の均一性，pH，無菌性，エンドトキシン／発熱性物質，不溶性微粒子，水分含量，抗菌性保存剤含量，抗酸化保存剤含量，溶出物，投与システムの機能性試験，浸透圧，粒子径分布(粒度)，再分散性，再調製時間
エアゾール剤*2	噴射時間と噴射量との関係，粒子径試験(懸濁タイプの場合)
エリキシル剤など*3	アルコール数測定
眼軟膏剤	金属性異物試験，無菌試験，放出試験，粒子径試験，展延性試験
硬膏剤などの経皮吸収剤	粘着性試験，放出試験
坐　剤	溶融温度試験，放出試験，軟化点
点眼剤	不溶性異物検査，無菌試験，放出試験，粒子径試験

＊1　剤形の特性によって異なるので，ケースバイケースの評価が必要
＊2　定量性が要求されるもの
＊3　酒精剤，チンキ剤，流エキス剤を含む

3．各剤形の特性に応じ評価すべき項目

剤形によって要求される機能が異なるので，ケースバイケースで評価される項目が存在する(**表2-3**)．本項目では最も汎用される経口固形製剤，経口液剤および注射剤に関して取り扱う．

a) 経口固形製剤

経口固形製剤，特に錠剤は患者自身が家庭で容易に服用でき，QOL の観点からも重要な剤形である．

錠剤は，有効成分が消化管で吸収され，全身循環を通って作用点に到達する．したがって，その錠剤が全身循環血液中の薬物濃度推移が適正な状態(効果を発揮するのに十分な薬物濃度推移かどうか臨床試験により確認されている)になるような品質特性を備えている必要がある．有効成分が消化管で吸収されるには，まず錠剤が崩壊し，その後，有効成分が溶出するプロセスを経る．薬物の血中濃度推移は，多くの場合この溶出速度に依存するので，製剤開発時に設計した適正な溶出－時間曲線(溶出プロファイル)をその錠剤の市販ロットで示すことが求められる．通常，開発段階で複数の試験液(水，酸性，弱酸性，中性)での溶出プロファイルを評価する．

溶出試験は製剤からの一定時間における原薬の放出量を試験液中の原薬濃度から評価する試験である．溶出性が規定の範囲内であることが確認できれば，生物学的に著しく非同等な製品が出荷されることを防止できると考えられている．

一方，溶出試験は1つの試験液の溶出曲線の1時点の結果を示しているに過ぎない．実際に各試験液での溶出プロファイルが適正であり，さらに服薬した患者が意図したバイオアベイラビリティ(BA)を示しているか否かは，溶出試験の結果だけではなく，

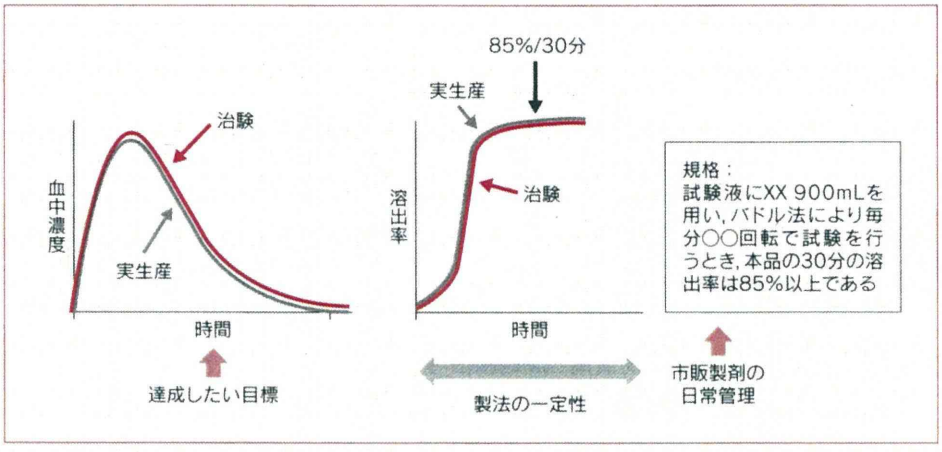

図2-1 ● 生物学的利用能，溶出プロファイルおよび溶出規格

製法の一定性が確保されて初めて保証される(**図2-1**).

即放錠の場合は通常1時点の評価を実施するが，徐放性製剤の場合は複数時点で評価する必要がある．崩壊過程が律速になり，速やかな溶出を示す場合には，溶出性の評価に代えて崩壊性を評価することも可能である．

また，投与単位の均一性の評価も重要である．この均一性の評価には，製剤の質量と製剤中の有効成分の含量の評価が行われる．近年は薬理活性の強い医薬品が開発され，1錠中の主薬含量が数%という薬剤も珍しくなく，そのようなケースでは製剤中の含量そのものの均一性(含量均一性)の評価が重要である．主薬含量が50%の製剤では最大でも2倍含量の錠剤しか存在しないが，2%の主薬含量の医薬品の場合，10倍含量の医薬品も想定可能であり，含量均一性の評価が重要となる．局方の含量均一性試験は，20個ないし30個を抜き取り試験するものであり，この試験により製剤均一性が保証されるには，実は製造プロセスで混合の均一性がきちんと制御されていることが条件となっている．

b) 経口液剤

経口液剤の場合は，錠剤よりも微生物汚染のリスクが高く，さらに化学的に不安定なので，微生物学的評価や安定化剤(酸化防止剤)の評価が重要である．

経口液剤には完全溶解型のタイプと懸濁型のタイプがあり，またそれぞれ用時溶解型の粉末剤(ドライシロップ)とあらかじめ溶解(懸濁)して患者に供給される製剤などさまざまな剤形がある．懸濁剤の場合は溶出性，粒子径分布，再分散性(沈降した薬剤を振盪して再分散するまでの時間)などを評価する必要がある．また粘稠な液剤の場合には，流動学的評価(粘度など)の評価が必要になる．

c) 注射剤

静脈注射剤は吸収プロセスを経ず，全身血液中に循環するために即効性が期待でき，しかも first path effect (初回通過効果)を受けないという利点をもつが，厳密な無菌性が要求される．さらに安全性の観点から，エンドトキシン／発熱性物質，不溶性微粒子

の評価など，多くの品質評価が必要である．

注射剤であっても，用時溶解型あるいは用時懸濁型の場合は投与単位の均一性として製剤均一性を評価しておく必要がある．ただし，凍結乾燥製剤は薬剤をいったん溶解後，バイアルに小分けしてから充填し凍結乾燥するので，各バイアル間の内容物は均一であり，質量偏差試験で評価可能である．

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Mass spectrometric glycoform profiling of the innovator and biosimilar erythropoietin and darbepoetin by LC/ESI-MS

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ABSTRACT

The recent patent expirations of erythropoietin (EPO) have promoted the development of biosimilars. Two and one biosimilar EPO products were approved in 2007 in Europe and in 2010 in Japan, respectively. Glycosylation heterogeneity of EPO is very complex, and its pattern has a large impact on its in vivo activity. In this study, glycoform profilings of biosimilar and innovator EPO products were performed using LC/ESI-MS. Glycoforms of EPO were detected within the range of m/z 1700–3600 at the 10^+–16^+ charge states. The charge-deconvoluted spectra showed complex glycoform mass profiles at 28,000–32,000 Da, and most of the observed peaks were assigned to the peptide (18,236 Da) + glycans with the compositions of $NeuAc_{10-14}Hex_{n+3}HexNAc_nFuc_3$ (n = 16–26) with or without some *O*-acetylations (+42 Da) and attachment of NeuGc for NeuAc or oxidation (+16 Da). Analysis of de-*N*-glycosylated EPO showed the distributions of *O*-glycans of $NeuAc_{1-2}Hex_1HexNAc_1$ and site occupancy. Each EPO product showed a characteristic glycoform profile with respect to sialylation, glycan size, *O*-acetylation of sialic acids and *O*-glycosylation. Analysis of darbepoetin suggested that glycans of darbepoetin were highly sialylated and *O*-acetylated. LC/ESI-MS was shown to be useful to evaluate the similarity of the glycoform profiles of EPO.

1. Introduction

Erythropoietin (EPO) is a glycoprotein hormone produced primarily by renal peritubular interstitial cells, and promotes the differentiation and development of red blood cells in the bone marrow [1–3]. The cDNA for EPO has been cloned [4,5], and recombinant human EPO products have been used for severe anemia therapy. EPO consists of 165 amino acid residues with three *N*-glycosylation sites at Asn24, Asn38, and Asn83, and one *O*-glycosylation site at Ser126. The *N*-linked glycans are bi-, tri- and tetra-antennary glycans, which typically terminate with sialic acid residues [6–8]. Asn38 and Asn83 contain mainly tetraantennary glycans with 0–3 *N*-acetyl lactosamine (LacNAc) structures, whereas Asn24 contains bi-, tri-, and tetra-antennary glycans with or without LacNAc structures [9–12]. The *O*-linked glycans were monosialyl trisaccharide and disialyl tetrasaccharide [6–8]. Because of the variable glycan structures, the product of EPO exists as a complex mixture of isoforms with different glycan structures attached. Glycans in EPO play roles in the in vitro and in vivo biological activities of EPO, the half-life of EPO in blood circulation, and the biosynthesis, secretion and stability of EPO [13–17]. Isoforms having higher sialic acid content show higher in vivo activity, and longer in vivo half-life. Sialylation of the preterminal galactose residues is required for preventing rapid clearance by hepatocytes, and tetraantennary *N*-glycan chains prevent EPO from renal clearance. The ratio of tetraantennary to biantennary glycans has a positive correlation with the in vivo activity of EPO [14]. On the other hand, *O*-linked glycan has no essential role in the in vivo or in vitro biological activity [16,17]. The recent patent expirations have promoted the development of biosimilars. Two and one biosimilar EPO products have been approved in Europe and Japan, respectively (Table 1). Because the structures and profiles of glycans attached to EPO have a large impact on its bioactivity, it is crucial to evaluate the similarity of the glycosylation profiles between biosimilar and innovator EPO products [18–20]. It is important to develop a set of methods to characterize the glycan heterogeneity [21].

Sialic acid analysis, and isoelectric focusing and capillary zone electrophoresis [18,20] have often been utilized for the evaluation of sialylated glycoproteins. Sialic acid analysis provides the contents of NeuAc and NeuGc, but does not give information on the glycan structures and glycoform distributions. It has been suggested that sialic acid content is insufficient for predicting the in vivo biological potency of recombinant EPO, and that examination of the branching structures and number of LacNAc repeats per *N*-glycan is also important for similarity/comparability evaluation

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Table 1
Epoetin products in Japan, Europe, and the USA.

Product name	*INN*	Approval		Cell	Comment
Innovator					
Epogen/Procrit	*epoetin alfa*	USA	June 1989	CHO	
Espo[a]		Japan	January 1990		
Eprex/Erypo[a]		EU			
Epogin[a]	*epoetin beta*	Japan	January 1990	CHO	
NeoRecormon		EU	July 1997		
Biopoin	*epoetin theta*	EU	October 2009	CHO	
Eporatio					
Biosimilar					
Epoetin alpha BS injection [JCR][a]	*epoetin kappa*	Japan	January 2010	CHO	Biosimilar to Espo
Binocrit		EU	August 2007	CHO	Biosimilar to Eprex/Erypo
Epostin alfa Hexal[a]					
Abseamed					
Silapo[a]	*epoetin zeta*	EU	December 2007	CHO	Biosimilar to Eprex/Erypo
Retacrit					

[a] Products tested in this study.

in glycosylation [22]. Isoelectric focusing and capillary zone electrophoresis provide charge isoform profiles based on the number of the attached sialic acid residues, but no information is available other than sialylation. Analysis of released glycans can provide information about the structure of glycans and its distribution [23,24]. However, the contents of the attached glycans and information about the glycoforms could not be elucidated. The glycan structures and their heterogeneity within individual EPO products are so complex that it has proven difficult to evaluate the differences in glycan profiles between samples. Analysis of glycopeptides provides not only information about the structure and distribution of the glycans, but also information about glycosylation sites. Due to the complex microheterogeneity, use of glycopeptide analysis for similarity evaluation is still challenging. Recently, glycoform profiling of biopharmaceuticals has been performed using mass spectrometry [25–27]. Mass spectrometric glycoform analysis can provide the masses of the glycoforms, which suggest the monosaccharide compositions of total attached glycans, and their distribution patterns, although it does not give information about the individual glycan structures and glycosylation sites. Mass spectrometry coupled to capillary electrophoresis has been shown to be a suitable analysis technique for the characterization of EPO glycoforms [28,29]. Meanwhile, MALDI TOF MS in comparison to ESI MS generally show lower resolution and accuracy in the higher mass range due to formation of adducts with salts, lower purity of available high-molecular-weight calibration standard, and/or fragmentation of glycan chain, such as loss of sialic acid residues [30,31]. Each of these methods has its own pros and cons; however, mass spectrometric glycoform analysis has the advantages of high sensitivity, provision of the mass information of total modifications, and fewer sample preparation steps. Due to the extreme complex glycosylation heterogeneity of EPO, glycoform profiles using mass spectrometry have not been shown to understand its characteristics just by looking. In this study, we performed mass spectrometric glycoform profiling using LC/ESI-MS to reveal the characteristics of glycoform profiles of innovator and biosimilar EPO products, and an erythropoietin analog, darbepoetin.

2. Materials and methods

2.1. Materials

Three innovator EPO products, Espo (*epoetin alpha*) from Kyowa Hakko Kirin Co., Ltd. (injection, 3000 IU), Epogin (*epoetin beta*) from Chugai Pharmaceutical Co., Ltd. (injection, 3000 IU) and Eprex (*epoetin alpha*) from Janssen-Cilag, Ltd. (injection, 3000 IU); three biosimilar products, epoetin alfa BS [JCR] (*epoetin kappa*) from Kissei Pharmaceutical Co., Ltd. (injection, 1500 IU), epoetin alfa Hexal from Hexal AG (injection, 3000 IU), and silapo (*epoetin zeta*) from Stada Arzneimittel AG (injection, 3000 IU); and one erythropoietin analog, NEPS (*darbepoetin alpha*) (injection, 15 μg) were used for this study. Glycopeptide *N*-glycosidase F (PNGase F; *Flavobacterium meningosepticum* expressed in *Escherichia coli*; EC 3.5.1.52) was purchased from Roche Diagnostics, and α-sialidase (*Arthrobacter ureafaciens*; EC 3.2.1.18) was from Sigma–Aldrich Co. LLC. All other reagents were of the highest quality available commercially. Milli-Q water was used in the preparation of samples and buffer solutions.

2.2. Glycoform profiling using LC/ESI-MS

2.2.1. Sample preparation

EPO products were diluted to 1500 IU/ml with 10 mM ammonium acetate, supplemented with 4 volumes of cold acetone, and kept at −20 °C for 2 h. The samples were centrifuged at 15,000 × g for 10 min at 4 °C. The supernatant was removed, and the pellet was dissolved in water. Aliquots of 60 IU were subjected to LC/ESI-MS. A 15 μg/ml aliquot of darbepoetin was treated in the same way.

2.2.2. Enzymatic digestion with α-sialidase

Acetone-precipitated EPO products (500 IU) were dissolved in 50 μl of sodium acetate buffer (10 mM, pH 5.5). This solution was incubated with 1 U of α-sialidase for 72 h at 37 °C.

2.2.3. Enzymatic digestion with PNGase F

Acetone-precipitated EPO products (500 IU) were dissolved in 50 μl sodium phosphate buffer (10 mM, pH 7.2) containing 5 mM ethylenediaminetetraacetic acid. This solution was supplemented with 1 U of PNGase F (1 U was defined as 1 μmol/min), and incubated for 72 h at 37 °C.

2.2.4. LC/ESI-MS analysis and data processing

A system of HPLC (Paradigm MS4, Michrome BioResources Inc.) and MS (Qstar Elite, AB Sciex Pte., Ltd.) equipped with a nanoESI ion source was used. The EPO sample was loaded onto a MonoCap C18 for Trap (0.2 mm × 150 mm; GL Sciences Inc., Tokyo, Japan) on the valve, and desalted twice using 20 μl of 0.1% trifluoroacetic acid. Then, the valve was switched, and the trap column was inversely connected on-line in a series with the analytical column. LC/ESI-MS was performed under the following conditions:

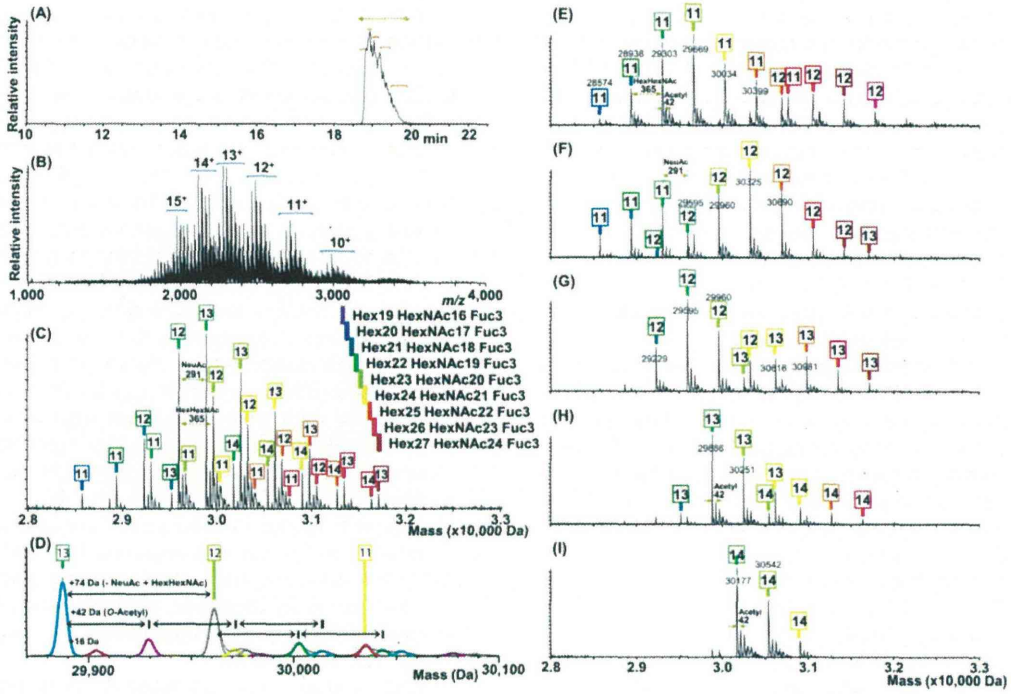

Fig. 1. Glycoform profiling of EPO A using reversed-phase LC/ESI-QTOF-MS. (A) Extract ion current chromatogram of *m/z* 1700–3600. (B) Integrated mass spectra at 18.7–20.0 min. (C) Deconvoluted spectrum. Deduced monosaccharide compositions of glycoforms are shown by the number (number of NeuAc residues) and color (compositions other than NeuAc). Peaks corresponding to *O*-acetylation of sialic acids (42 Da higher mass) and NeuGc for NeuAc or oxidation (16 Da higher mass) were also detected. (D) The range of *m/z* 29,870–30,100 was magnified. Overlapping peaks were resolved by curve-fitting. (E)–(I) Deconvoluted spectra at 18.7–18.9, 18.9–19.0, 19.0–19.2, 19.2–19.5, and 19.5–20.0 min, respectively.

column, MonoCap C18 fast-flow column (0.1 mm × 250 mm; GL Sciences Inc.); eluate A, 0.1% formic acid; eluate B, 0.1% formic acid/acetonitrile; gradient conditions, 0–2 min 10% B, 2–22 min 10–80% B; flow rate, 0.5 μl/min; nanospray voltage, 2400 V; *m/z* range, 1000–4000. In order to obtain the glycoform profiles, the mass spectra throughout the entire elution period were integrated, and then deconvoluted using the Bayesian Protein Reconstruct tool in the BioAnalyst ver. 1.1 software package with the following parameters: mass range, 20,000–40,000; mass step, 1; S/N = 0; *m/z* range, 1700–3600; adduct, proton; iteration, 50. In the case of desialylated or de-*N*-glycosylated EPO, the mass range and *m/z* range were changed accordingly. In order to obtain the peak intensity, a commercial curve fitting software package, PeakFit ver. 4.11 (Systat Software Inc., San Jose, CA) was used against the deconvoluted mass spectrum. Text files containing a list of mass values and intensity values were imported to PeakFit software. The initial peak location was determined by the Autofit Peaks I Residuals option, and then manually for minor peaks. Then, peak fitting was performed without smoothing and baseline-subtraction. Mass spectrometric glycoform analysis was performed under repeatability conditions (2 days, 2 replicates) ($n = 4$) for each EPO product.

3. Results and discussion

3.1. Mass spectrometric glycoform profiling of EPO product A

EPO products were analyzed by LC/ESI-MS to obtain mass spectrometric glycoform profiles. As a representative example, the data of innovator EPO product A (EPO A) are shown in Fig. 1. EPO A was eluted for 18.7–20.0 min, as indicated by the extracted ion current chromatogram at *m/z* 1700–3600 (Fig. 1A), and detected at an *m/z* about 1700–3600 with the 10^+–16^+ charge states (Fig. 1B). The charge-deconvolved mass spectrum showed many peaks at 28,500–32,000 Da, and the different mass values, such as 365 Da (HexHexNAc) and 291 Da (NeuAc), suggested glycosylation heterogeneity (Fig. 1C). Most of these peaks could be assigned to the peptide (18,235.7 Da) + glycans with the compositions of $NeuAc_{11-14}Hex_{n+3}HexNAc_nFuc_3$ ($n = 16$–24) [28]. These findings are consistent with the reports that attached *N*-glycans of recombinant human EPO were of core-fucosylated bi-, tri-, and tetra-antennary complex-type and highly capped with sialic acids, and that *O*-glycans were disaccharides of GalGalNAc with one or two sialic acids [6–8]. To show the glycoform assignment clearly, these peaks were annotated using tags, in which the number and color indicated the number of the NeuAc residues and monosaccharide compositions other than NeuAc of total attached glycans, respectively. Here, glycoforms with three of the core-fucosylated tetraantennary *N*-linked glycans without LacNAc and one of *O*-glycan gave the neutral monosaccharide compositions, $Hex_{22}HexNAc_{19}Fuc_3$ and 14 of the maximum attachable number of NeuAc. Peaks with 42 Da higher mass were also observed, and this may indicate *O*-acetylation of sialic acids. The small peaks with ~16 Da higher mass, which were not completely resolved, might indicate the attachment of NeuGc for NeuAc and/or the oxidation of the protein portion. It should be noted that if there were only a few percent of *O*-acetylation in sialic acids or a few percent of NeuGc contents, glycoforms with these modifications would be observed at about 10% of the glycoforms without modification, because molecules have 11–14 sialic acid residues. Since almost all peaks could be assigned based on the masses of the glycan moiety, the peptide portion would be highly homogeneous.

In ESI-MS, each glycoform produces a series of peaks with multiple charge states in the spectrum resulting from the electrospray ionization process. In order to obtain their original mass information, they have to be deconvoluted into zero-charged masses. Deconvolution parameters could affect the mass spectrometric

glycoform profiles. It is well known that relatively high artifact peaks will appear at half and twice the molecular mass in deconvoluted mass spectra. Noise peaks and peaks unrelated to EPO in the mass spectra might cause artifact peaks in deconvoluted mass spectra. The glycan structure can affect the pattern of charge state distribution. Thus, the entire set of EPO ions at m/z 1700–3600 was used for deconvolution, and the range for the resultant masses was set to be 20,000–40,000 Da to prevent the peaks at half and twice of the molecular masses. Although the mass spectra were extremely complex, the Bayesian Protein Reconstruct tool successfully gave a probable pattern of deconvoluted mass spectra.

Multiple *O*-acetylation on NeuAc made the mass spectra complicated. Glycoforms with one less NeuAc (−291 Da) and one more HexHexNAc (+365 Da) had 74 Da higher masses, and glycoforms with two *O*-acetylations had 84 Da higher masses. These peaks overlapped each other. In order to show the glycoform pattern objectively, relative peak intensity should be determined. Because the Bayesian Protein Reconstruct tool provides the peak intensity only for the major glycoform peaks, but not for the minor peaks or overlapping peaks, we used a commercial curve fitting software package, PeakFit ver. 4.11, to calculate the peak intensity (D). The relative peak intensities were summarized in Table 2, as described below. The glycoforms of $NeuAc_{12}Hex_{22}HexNAc_{19}Fuc_3$ (29,596.9 Da) were taken as 100 (%).

The glycoforms of EPO were slightly separated by HPLC according to the number of sialic acids and the size of glycans. Deconvoluted mass spectra during 18.7–18.9, 18.9–19.0, 19.0–19.2, 19.2–19.5, and 19.5–20.0 min (Fig. 1E–I) indicated that glycoforms with fewer sialic acids eluted much earlier, and then the glycoforms with larger glycans eluted slightly earlier. *O*-Acetylation seemed to have little effect on the retention times. The deconvoluted mass spectra from short elution periods showed minor glycoforms such as *O*-acetylation and NeuGc or protein oxidation more clearly.

3.2. Comparison of mass spectrometric glycoform profiles between EPO products

Mass spectrometric glycoform profiling of 3 innovator products (EPO A, B, and C) and 3 biosimilar products (EPO A′, C′, and C″) was performed under the same conditions as used for the profiling of EPO A, and representative deconvoluted mass spectra are shown in Fig. 2A–F (Fig. 2A is the same as Fig. 1C). Here, EPO A′ is a biosimilar product developed using EPO A as a reference product, and EPO C′ and EPO C″ are biosimilar products developed using EPO C as a reference product. Almost all the observed peaks could be assigned as glycoforms with monosaccharide compositions $NeuAc_{9-14}Hex_{n+3}HexNAc_nFuc_3$ (n = 16–26) as described in Fig. 1, except for the unusual peaks in EPO C′ (described below). All the EPO products showed different glycoform profiles.

In EPO A, the number of sialic acids attached to one molecule ranged from 11 to 14, and the glycoforms with 12 and 13 sialic acids were most abundant. *O*-Acetylations of NeuAc were observed. EPO A′ showed lower sialylation compared to EPO A; the number of attached sialic acids ranged from 9 to 14, with the glycoforms having 11 and 12 sialic acids being most abundant. The wide range of glycoforms having a mass of 365 Da (HexHexNAc unit) indicated that EPO A′ contained more LacNAc structures. In Epo B, the number of attached sialic acids ranged from 10 to 14, and the glycoforms with 12 and 13 sialic acids were most abundant. The glycoforms with 14 sialic acids were relatively abundant than in the other products. No *O*-acetylation was observed. Attachment of NeuGc and/or oxidation was observed more frequently than in the other products. EPO C showed glycoform profiles similar to EPO A, but its level of sialylation was slightly lower and there were a few more of glycoforms with lower mass. The glycoforms with 12 sialic acids were most abundant and the number of glycoforms with 14 sialic acids was very low. *O*-Acetylation was observed most among the EPO products used in this study. In the EPO C′, unusual peaks (27,973, 28,338 and 28,704 Da) were observed in the lower mass region. It is known that EPO C′ contains high-mannose-type glycans with mannose 6-phosphate modification at Asn24 [32]. If one of the *N*-linked glycans were high-mannose-type glycans, then the maximum number of attached sialic acids would be 10, as calculated from the two tetrasialo-tetraantennary glycans for *N*-linked glycan and disialylated GalGalNAc for *O*-linked glycan. Taking this information into account, the monosaccharide compositions of these peaks were suggested to be $NeuAc_{10}Hex_{20+n}HexNAc_{15+n}Fuc_2Man6P$ (n = 0–2) [29]. These findings also suggested that the monosaccharide compositions of high-mannose-type glycan were $Hex_5HexNAc_2M6P$. Although $NeuAc_{12}Hex_{17+n}HexNAc_{15+n}Fuc_3$ (n = 0–2) also had the same mass, the data from α-sialidase digestion supported the former monosaccharide compositions (described below). When the glycoforms containing high-mannose-type glycan were excluded, the number of attached sialic acids ranged from 11 to 14, and the glycoforms with 12 and 13 sialic acids were most abundant. Relatively higher *O*-acetylation was observed. In EPO C″, the number of attached sialic acids ranged from 10 to 14, the glycoforms with 11 sialic acids were most abundant, and there were few glycoforms with 14 sialic acids. There were few glycoforms with *O*-acetylation and NeuGc and/or oxidation.

All EPO products were analyzed 4 times under repeatability conditions, and the relative intensity of glycoform peaks and standard deviations are summarized in Table 2. It is important that glycoforms with the same mass or the same monosaccharide compositions were a mixture of different isomers with different patterns of branching, addition of LacNAc, sialylation, and site-specificity, and that the compositions of these isomers were characteristic to the products. Therefore, the overall pattern of glycoform profiles would be important for the evaluation of similarity. The relative standard deviations of the relative peak intensities were around or less than 10% for major-to-moderate glycoforms. Despite the complex data processing, such as deconvolution and curve fitting, glycoform profiling using LC/ESI-MS showed a certain level of analytical repeatability. The distributions of the number of sialylations per molecule were calculated, and are also shown at the bottom of Table 2. The highest frequency of 14 sialylated glycoforms was observed in EPO B and EPO C′. The calculated average number of sialylations was 12.5, 11.7, 12.3, 12.0, 12.3, and 11.4 for EPO A, A′, B, C, C′, and C″, respectively.

3.3. Comparison of mass spectrometric de-sialylated glycoform profiles between EPO products

Since the glycoform profiles of EPO were highly complex, profiling of de-sialylated EPO was performed to confirm the monosaccharide compositions of the asialo-glycan structures and their distribution (Fig. 3). The profiles showed the distribution with respect to branching of *N*-glycan and addition of LacNAc structures. Almost all peaks were assigned to monosaccharide compositions, $Hex_{n+3}HexNAc_nFuc_3$; n = 16–24 or 25 in EPO A, EPO B, EPO C and EPO C′, and n = 16–27 in EPO A′. The most abundant de-sialylated glycoforms were n = 19 in EPO A, EPO B, EPO C, and EPO C′, but n = 21–22 in EPO A′, and n = 19–20 in EPO C″, respectively. If EPO was attached by three core-fucosylated tetraantennary glycans without LacNAc for *N*-linked glycan and GalGalNAc for *O*-linked glycan, the monosaccharide compositions of total glycans were $Hex_{22}HexNAc_{19}Fuc_3$ (26,101.9 Da). Peaks lower than this value indicated less branching, and peaks higher than this indicated more LacNAc structures. Unusual peaks (25,059, 25,425 and 25,790 Da) were observed only in EPO C′. These were assigned to the composition $Hex_{20+n}HexNAc_{15+n}Fuc_2Man6P$

Table 2
Summary of glycoform profiles of innovator and biosimilar EPO products in Japan and Europe.

Deduced monosaccharide compositions of glycoforms						Calculated mass (Da)	Relative peak intensity (%)[a]					
NeuAc	Man6P	Hex	HexNAc	dHex	O-Acetyl		EPO A	EPO A′	EPO B	EPO C	EPO C′	EPO C″
0	0	0	0	0	0	18,235.7						
10	1	20	15	2	0	27,973.4					69.8 ± 5.4	
					1	28,015.4					20.6 ± 2.0	
					2	28,057.4					21.5 ± 1.3	
					3	28,099.5					17.5 ± 1.1	
					4	28,141.5					9.0 ± 0.8	
10	1	21	16	2	0	28,338.7					51.6 ± 2.6	
					1	28,380.7					14.2 ± 0.8	
					2	28,422.8					12.8 ± 2.4	
10	1	22	17	2	0	28,704.0					17.8 ± 2.0	
10		19	16	3	0	27,918.4			5.0 ± 0.1			44.8 ± 4.9
					1	27,960.5						2.9 ± 1.6
9		20	17	3	0	27,992.5			2.8 ± 0.6			
10		20	17	3	0	28,283.8		18.3 ± 2.9	14.6 ± 0.9			92.2 ± 6.9
11		20	17	3	0	28,575.0	8.6 ± 0.7	24.6 ± 1.5	31.1 ± 2.4	23.6 ± 1.3	41.6 ± 1.3	110.5 ± 8.8
					1	28,617.0				5.9 ± 0.3	5.6 ± 0.6	10.0 ± 1.0
					2	28,659.1				14.0 ± 2.0		
					3	28,701.1				1.3 ± 0.0		
12		20	17	3	0	28,866.3		5.5 ± 1.1	14.3 ± 0.9		69.8 ± 5.4	5.5 ± 0.1
					1	28,908.3					18.3 ± 1.8	
					2	28,950.3					25.9 ± 3.0	
9		21	18	3	0	28,357.8			0.4 ± 0.3			
10		21	18	3	0	28,649.1		50.3 ± 2.7	16.5 ± 0.9			90.2 ± 2.8
11		21	18	3	0	28,940.3	19.7 ± 1.1	63.7 ± 3.7	54.9 ± 4.6	56.7 ± 3.2		**167.2 ± 13.3**
					1	28,982.4	0.6 ± 0.3		0.4 ± 0.0	18.0 ± 1.1		
					2	29,024.4				22.8 ± 2.8		
					3	29,066.5				9.2 ± 1.4		
					4	29,108.5				7.2 ± 0.9		
12		21	18	3	0	29,231.6	41.9 ± 1.9	24.9 ± 4.8	71.4 ± 1.0	40.1 ± 3.4	**112.2 ± 6.0**	60.1 ± 3.5
					1	29,273.6	7.5 ± 0.6			15.7 ± 2.5	32.2 ± 1.1	3.6 ± 1.1
					2	29,315.7	6.5 ± 0.8			40.1 ± 5.5	32.8 ± 1.7	
					3	29,357.7				18.7 ± 1.6	5.9 ± 1.2	
					4	29,399.7				19.0 ± 2.8		
					5	29,441.8				5.2 ± 1.2		
13		21	18	3	0	29,522.9	7.5 ± 1.1		30.6 ± 3.6		39.2 ± 4.7	
					1	29,564.9					3.4 ± 0.8	
					2	29,606.9	9.6 ± 0.6				25.8 ± 2.2	
					3	29,649.0	2.1 ± 0.4				6.0 ± 1.5	
					4	29,691.0	2.5 ± 0.2					
9		22	19	3	0	28,723.2		25.8 ± 2.5				
10		22	19	3	0	29,014.4		63.8 ± 3.8	10.7 ± 1.1			59.7 ± 5.0
					1	29,056.5						9.1 ± 1.8
11		22	19	3	0	29,305.7	26.1 ± 0.9	90.7 ± 5.0	45.2 ± 2.9	72.7 ± 4.9		163.8 ± 12.5
					1	29,347.7	6.9 ± 0.6	15.7 ± 1.7		28.3 ± 1.9		16.6 ± 4.4
					2	29,389.7	2.4 ± 0.2			30.7 ± 3.5		
					3	29,431.8				10.3 ± 1.9		
12		22	19	3	0	29,596.9	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	**100.0 ± 0.0**	100.0 ± 0.0	100.0 ± 0.0
					1	29,639.0	22.2 ± 0.8			42.1 ± 0.3	23.7 ± 0.9	5.0 ± 0.3
					2	29,681.0	15.9 ± 1.6			57.0 ± 3.5	20.9 ± 2.0	
					3	29,723.0	4.2 ± 0.7			30.0 ± 1.6	3.6 ± 0.6	
					4	29,765.1				20.8 ± 1.3		
13		22	19	3	0	29,888.2	**108.1 ± 4.1**	48.1 ± 7.9	**130.0 ± 6.5**	65.5 ± 4.4	92.4 ± 11.7	30.6 ± 7.0
					1	29,930.2	23.4 ± 0.5		2.8 ± 0.3	22.5 ± 0.5	26.9 ± 3.3	
					2	29,972.3	20.6 ± 1.4			44.5 ± 3.8	35.7 ± 4.4	
					3	30,014.3	10.5 ± 1.5			30.5 ± 3.7	17.5 ± 2.0	
					4	30,056.3	8.1 ± 1.1			34.3 ± 2.8		
					5	30,098.4				19.7 ± 5.5		
14		22	19	3	0	30,179.4	27.3 ± 3.6		68.9 ± 8.4	15.3 ± 2.0	33.3 ± 4.8	
					1	30,221.5	3.6 ± 0.6				9.3 ± 2.1	
					2	30,263.5	9.7 ± 0.8				19.5 ± 3.2	
					3	30,305.6	5.1 ± 0.7				14.9 ± 0.8	
					4	30,347.6	5.4 ± 1.3				16.2 ± 3.3	
10		23	20	3	0	29,379.8		72.7 ± 2.6	6.6 ± 0.7			30.0 ± 4.2
					1	29,421.8		9.2 ± 2.3				5.2 ± 1.0
11		23	20	3	0	29,671.0	22.0 ± 1.0	138.7 ± 4.8	30.8 ± 2.3	50.6 ± 1.4		132.3 ± 6.0
					1	29,713.0	9.2 ± 1.6	9.6 ± 1.3		21.4 ± 3.5		11.9 ± 3.5
					2	29,755.1	2.8 ± 0.3			15.8 ± 1.9		
					3	29,797.1				7.3 ± 1.0		
12		23	20	3	0	29,962.3	76.4 ± 1.9	125.6 ± 11.4	63.8 ± 2.0	72.0 ± 6.9	63.7 ± 5.2	113.7 ± 5.9
					1	30,004.3	25.1 ± 3.1	8.9 ± 2.2		31.4 ± 3.4	20.9 ± 2.8	12.4 ± 1.0
					2	30,046.3	15.8 ± 1.7			44.6 ± 4.5	16.0 ± 1.2	
					3	30,088.4	4.5 ± 0.6			18.6 ± 5.6	3.8 ± 0.2	
					4	30,130.4				16.6 ± 6.4		

Table 2 (*Continued*)

Deduced monosaccharide compositions of glycoforms						Calculated mass (Da)	Relative peak intensity (%)[a]					
NeuAc	Man6P	Hex	HexNAc	dHex	O-Acetyl		EPO A	EPO A′	EPO B	EPO C	EPO C′	EPO C″
13		23	20	3	0	30,253.5	84.3 ± 3.9	72.7 ± 6.9	81.9 ± 2.5	43.7 ± 1.2	76.8 ± 6.5	44.5 ± 9.3
					1	30,295.6	21.7 ± 2.1			17.0 ± 2.1	26.0 ± 4.0	
					2	30,337.6	15.0 ± 0.8			31.6 ± 3.1	29.4 ± 5.1	
					3	30,379.6	8.0 ± 0.8			19.8 ± 1.8	17.5 ± 0.6	
					4	30,421.7					11.2 ± 0.8	
14		23	20	3	0	30,544.8	23.6 ± 3.6	8.6 ± 3.4	42.6 ± 6.1	7.7 ± 1.0	33.3 ± 3.8	
					1	30,586.8	3.4 ± 0.3				11.9 ± 1.8	
					2	30,628.8	5.3 ± 0.5				16.4 ± 1.6	
10		24	21	3	0	29,745.1		69.8 ± 7.5				
11		24	21	3	0	30,036.3	15.9 ± 1.3	131.3 ± 4.7	18.1 ± 1.0	34.0 ± 3.7		86.6 ± 4.8
					1	30,078.4	6.8 ± 1.0	19.8 ± 3.3		13.4 ± 1.8		10.5 ± 2.1
					2	30,120.4				8.5 ± 3.0		
12		24	21	3	0	30,327.6	56.0 ± 1.2	144.9 ± 14.2	42.2 ± 3.0	49.8 ± 2.7	38.5 ± 5.3	111.1 ± 7.7
					1	30,369.6	18.9 ± 1.5	9.9 ± 2.1		19.5 ± 0.7	16.7 ± 1.8	11.5 ± 2.0
					2	30,411.7	11.0 ± 1.5			20.0 ± 1.1	14.2 ± 3.1	
					3	30,453.7	3.3 ± 0.4			14.3 ± 4.2		
					4	30,495.7				9.2 ± 0.3		
13		24	21	3	0	30,618.9	62.1 ± 3.2	89.6 ± 10.8	58.9 ± 2.2	35.1 ± 1.9	66.6 ± 4.9	60.5 ± 4.7
					1	30,660.9	14.0 ± 0.9			9.2 ± 2.5	22.1 ± 1.4	
					2	30,702.9	10.7 ± 0.4			19.3 ± 0.8	26.2 ± 5.2	
					3	30,745.0	3.7 ± 0.3					
14		24	21	3	0	30,910.1	16.2 ± 3.4	19.3 ± 3.2	28.6 ± 4.7		25.5 ± 2.8	
					1	30,952.1	1.6 ± 0.2				3.1 ± 1.1	
					2	30,994.2	1.4 ± 0.2				13.0 ± 3.4	
10		25	22	3	0	30,110.4		53.8 ± 4.5	1.7 ± 0.4			
11		25	22	3	0	30,401.7	9.3 ± 1.0	114.2 ± 8.3	9.8 ± 0.5	12.8 ± 1.9		49.4 ± 4.1
					1	30,443.7	2.9 ± 0.8	10.3 ± 1.2				5.7 ± 0.7
					2	30,485.7	2.2 ± 0.3					3.2 ± 0.8
12		25	22	3	0	30,692.9	33.9 ± 1.4	**147.5 ± 10.6**	25.1 ± 2.3	32.1 ± 1.4	14.7 ± 3.1	88.0 ± 6.1
					1	30,735.0	9.1 ± 1.0	12.0 ± 2.1		7.5 ± 1.4		7.7 ± 3.6
					2	30,777.0				11.4 ± 5.5		
					3	30,819.0				4.4 ± 0.4		
13		25	22	3	0	30,984.2	40.3 ± 1.3	100.2 ± 9.2	35.1 ± 0.9	18.2 ± 2.7	39.3 ± 4.2	52.4 ± 4.0
					1	31,026.2	6.1 ± 1.0	5.6 ± 0.8		±	8.6 ± 0.7	
					2	31,068.3	4.8 ± 1.6			11.8 ± 0.9	14.3 ± 2.3	
					3	31,110.3	1.4 ± 0.1			±		
14		25	22	3	0	31,275.4	10.1 ± 1.3	24.2 ± 8.1	18.4 ± 2.3	6.8 ± 0.6	14.6 ± 1.5	
					1	31,317.5					5.2 ± 1.3	
					2	31,359.5						
10		26	23	3	0	30,475.8		6.9 ± 0.0				
11		26	23	3	0	30,767.0		90.8 ± 5.7	4.9 ± 0.3			27.1 ± 4.0
					1	30,809.0		6.7 ± 0.1				2.9 ± 1.1
12		26	23	3	0	31,058.3	16.4 ± 1.0	128.5 ± 5.7	11.6 ± 1.0	15.1 ± 1.4	9.7 ± 3.1	48.0 ± 3.7
					1	31,100.3	3.7 ± 0.3					
					2	31,142.3	2.3 ± 0.4					
13		26	23	3	0	31,349.5	17.2 ± 1.0	101.1 ± 6.3	17.3 ± 1.0		13.0 ± 1.1	37.8 ± 1.8
					1	31,391.6	1.5 ± 0.1	7.6 ± 0.6				3.0 ± 0.7
14		26	23	3	0	31,640.8	3.1 ± 0.8	36.0 ± 1.6	9.9 ± 1.3		6.5 ± 1.1	5.0 ± 0.8
					1	31,682.8						
					2	31,724.8	0.7 ± 0.2				4.9 ± 0.1	
11		27	24	3	0	31,132.3		66.4 ± 2.6	0.5 ± 0.1			
12		27	24	3	0	31,423.6		94.4 ± 3.4	1.4 ± 0.4			
13		27	24	3	0	31,714.9	9.1 ± 0.5	85.4 ± 5.2	8.4 ± 0.7	11.1 ± 2.3	8.7 ± 0.7	28.3 ± 1.0
					1	31,756.9		13.3 ± 3.1				
14		27	24	3	0	32,006.1		6.7 ± 1.9	2.1 ± 0.1			
11		28	25	3	0	31,497.7		41.8 ± 4.1				
12		28	25	3	0	31,788.9		54.8 ± 14.5				
13		28	25	3	0	32,080.2		27.4 ± 5.7				

Distribution of glycoforms based on the sialylation (%)		EPO A	EPO A′	EPO B	EPO C	EPO C′	EPO C″
NeuAc	M6P						
10	1	0.0	0.0	0.0	0.0	13.3	0.0
9		0.0	1.0	0.3	0.0	0.0	0.0
10		0.0	12.8	4.9	0.0	0.0	17.0
11		11.1	30.6	17.5	27.6	2.7	40.7
12		38.9	31.8	29.5	44.9	36.6	28.9
13		40.4	20.4	32.6	25.8	34.5	13.1
14		9.6	3.5	15.2	1.8	12.9	0.3
Calculated average no. of NeuAc per molecule		12.5	11.7	12.3	12.0	12.3	11.4

[a] Peak intensity of glycoform $NeuAc_{12}Hex_{22}HexNAc_{19}Fuc_3$ was considered 100%.

The most abundant glycoforms are shown in bold.

Glycoforms with NeuGc and/or oxidation were not included.

Differences between calculated mass and observed mass were within 3 Da (data not shown).

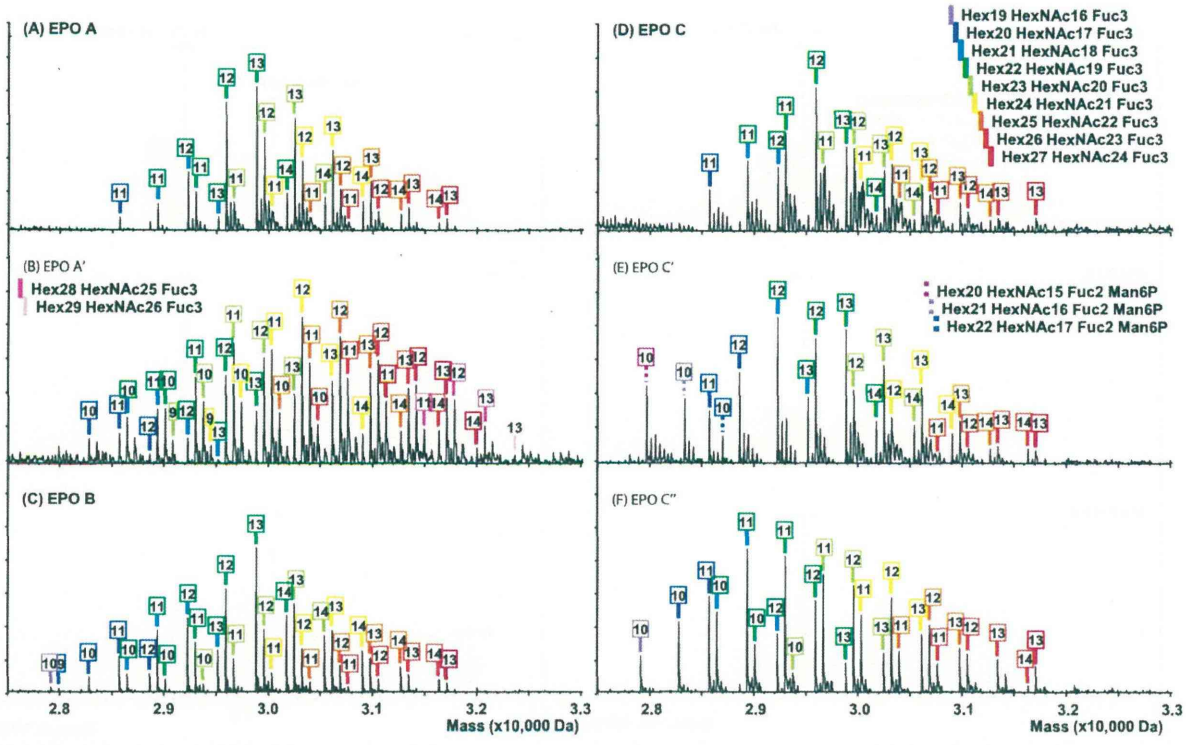

Fig. 2. Comparison of mass spectrometric glycoform profiles of innovator and biosimilar erythropoietins. (A) EPO A, (C) EPO B and (D) EPO C are innovator products. (B) EPO A′ is a biosimilar product to EPO A, and (E) EPO C′ and (F) EPO C″ are biosimilar products to EPO C.

(n = 0–2), which would be derived from the glycoforms with $NeuAc_{10}Hex_{20+n}HexNAc_{15+n}Fuc_2Man6P$ (n = 0–2). These findings agreed with the data of glycoform profiling.

In the de-sialylated glycoforms, peaks with 42 Da higher mass were not detected. This indicated the increase of 42 Da mass was due to *O*-acetylation of sialic acid. Peaks with 16 Da higher mass were detected at higher levels before digestion in all samples. This finding would have been due to oxidization of the protein portion during α-sialidase digestions, because the intensities of these peaks increased over time (data not shown). This suggested that one easily

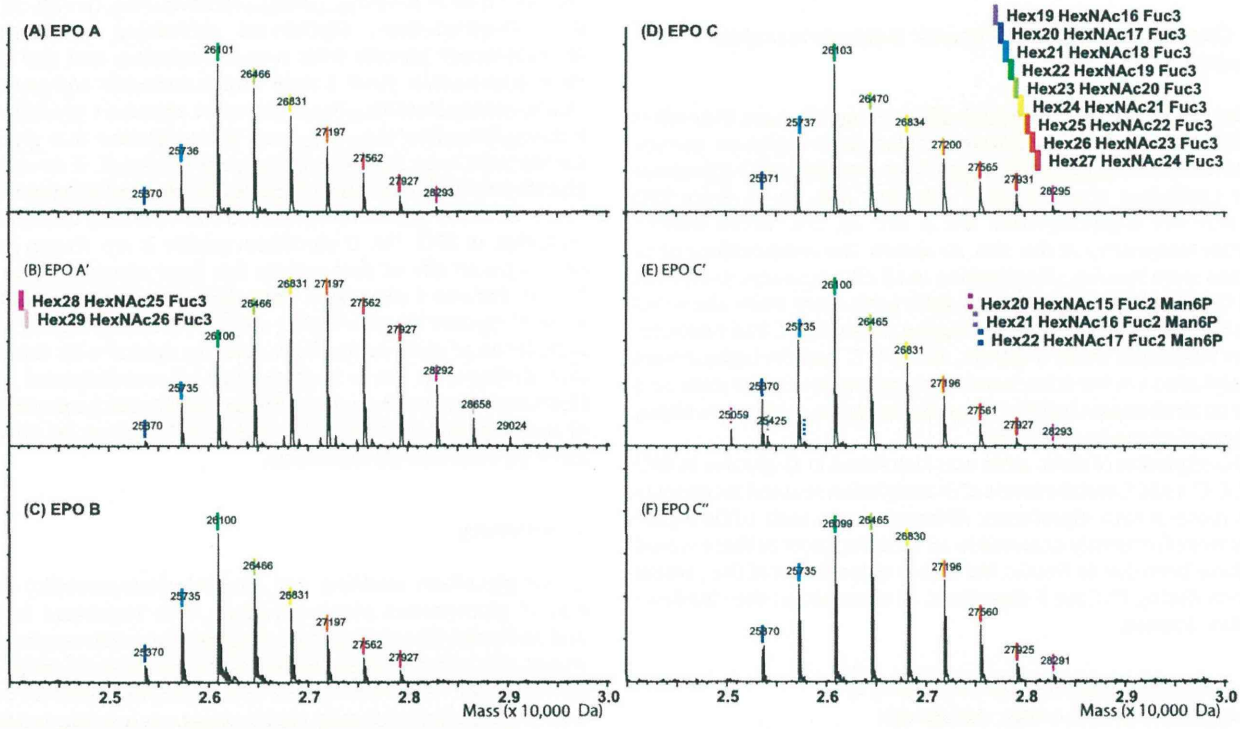

Fig. 3. Comparison of mass spectrometric de-sialylated glycoform profiles of innovator and biosimilar erythropoietins. (A–F) Innovator products EPO A, B and C, and biosimilar products EPO A′, C′, and C″, respectively. Peaks with 16 Da higher masses would be due to oxidation of the protein portion during α-sialidase digestion.

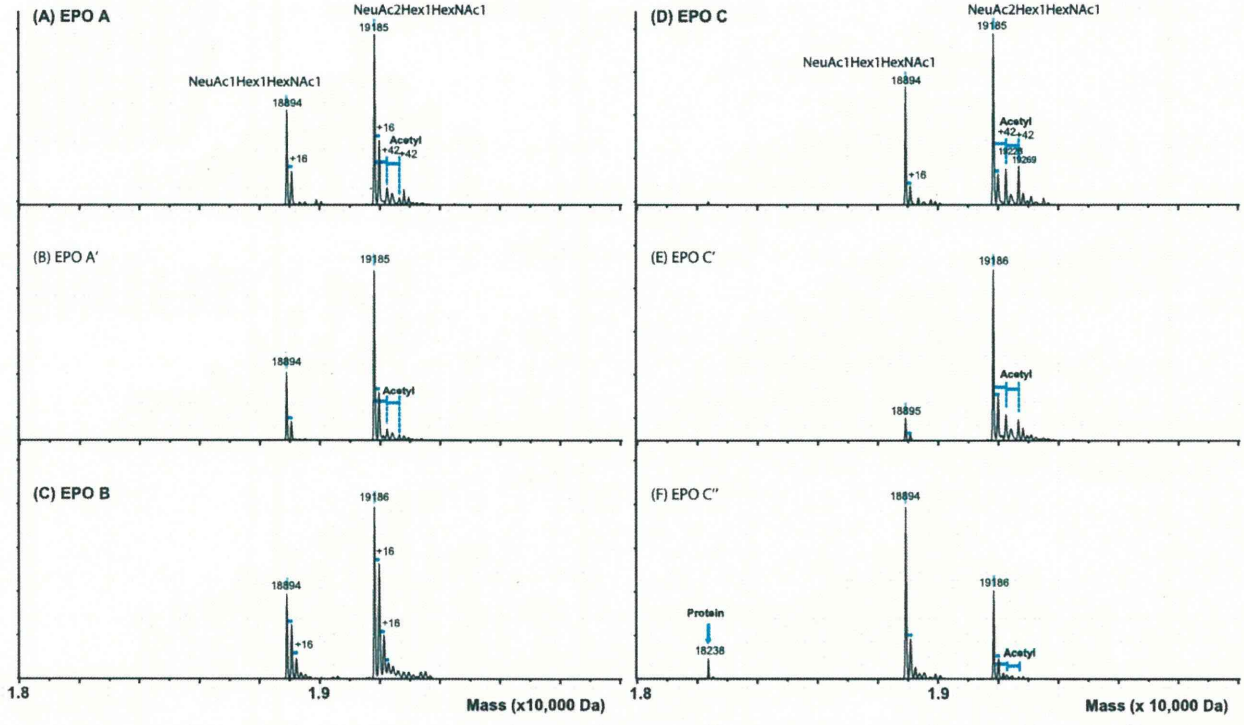

Fig. 4. Comparison of mass spectrometric *O*-linked glycoform profiles of innovator and biosimilar erythropoietins. (A)–(F) Innovator products EPO A, B and C, and biosimilar products EPO A′, C′, and C″, respectively. Peaks with 16 Da higher masses would be due to oxidation of the protein portion during PNGase F digestion.

oxidizable site may be present in the erythropoietin structure. In order to determine the contents of NeuAc and NeuGc, sialic acid analysis would be needed. It is interesting that peaks with 32 Da higher mass were more obvious in EPO B. This may suggest that EPO B might be more susceptible to oxidation.

3.4. Comparison of mass spectrometric O-glycoform profiles between EPO products

Because *O*-glycosylation of the EPO is thought to have little effect on EPO efficacy, it is important to reveal how *O*-glycans participated in the total glycoform profiles. Thus, profilings of *O*-glycoform were conducted after PNGase F digestion (Fig. 4A–F). Since EPO has only one *O*-glycosylation site at Ser126, this profile showed microheterogeneity at this site. As shown, the compositions of *O*-glycans were $NeuAc_{1-2}HexHexNAc$ in all EPO products. Except for EPO C″, *O*-glycoforms with two sialic acids were more abundant than those with one sialic acid. Among these, EPO C had relatively lower sialylation on its *O*-glycan, and EPO C′ had the highest level of sialylation. On the other hand, *O*-glycoforms with one sialic acid were more abundant in EPO C″, and, furthermore, molecule without *O*-glycosylation was observed.

O-acetylation of sialic acids was also found at *O*-glycans in EPO A, A′, C, C′ and C″, and the levels of *O*-acetylation seemed to correlate with those in total glycoforms. Although peaks with 16 Da higher mass were frequently detected in all samples, most of these would not have been due to NeuGc, but rather to oxidation of the protein portion during PNGase F digestions, as observed in the sialidase-treated samples.

3.5. Mass spectrometric glycoform profiling of a hyperglycosylated EPO analog, darbepoetin

Darbepoetin is an EPO analog that was developed by site-directed mutagenesis to introduce two additional *N*-glycosylation sites for longer serum half-lives [33]. Darbepoetin was analyzed by LC/ESI-MS, and the deconvoluted mass spectrum is shown in Fig. 5A. There were many peaks differing by 42 Da due to heavy *O*-acetylation. Almost all peaks could be assigned to the peptide (18,176.6 Da) + glycans with the compositions of $NeuAc_{19-22}Hex_{n+5}HexNAc_nFuc_5$ (n = 29–32) with 0–13 *O*-acetylations. Glycoforms containing five tetrasialo-tetraantennary glycans with core-fucosylation, and one disialylated HexHexNAc yield a total monosaccharide composition of $NeuAc_{22}Hex_{36}HexNAc_{31}Fuc_5$. The most abundant glycoform was $NeuAc_{22}Hex_{36}HexNAc_{31}Fuc_5$, and this suggested that glycans of darbepoetin were highly branched and sialylated. A de-sialylated glycoform profile is shown in Fig. 5B. This showed that heterogeneity in the asialo-glycan compositions was relatively low compared with that in EPO. The *O*-glycoform profile is not shown because one *N*-glycan site of darbepoetin has been reported to be resistant to PNGase F digestions [30]. Darbepoetin has not only two more *N*-glycans but also higher sialylation and extremely high *O*-acetylation of sialic acids. These findings agreed with the reports that darbepoetin has a large number of tetrasialylated glycans (tetraantena, triantena and biantenna with LacNAc) and a high level of *O*-acetylation of sialic acids [34]. LC/ESI-MS can be applied to more glycosylated glycoproteins.

4. Summary

For glycoform profiling and similarity/comparability evaluation of glycoprotein pharmaceuticals, it is important to detect and understand how the glycan structures or substructures, which would affect the efficacy and safety, change. Recently, MS of intact glycoproteins was considered as useful analytical tools for evaluation of glycan heterogeneity. In this study, we investigated the use of LC/ESI-MS to characterize and compare glycoforom profiles of innovator and biosimilar EPO products. Glycoform profiling using LC/ESI-MS successfully revealed the characteristics of innovator

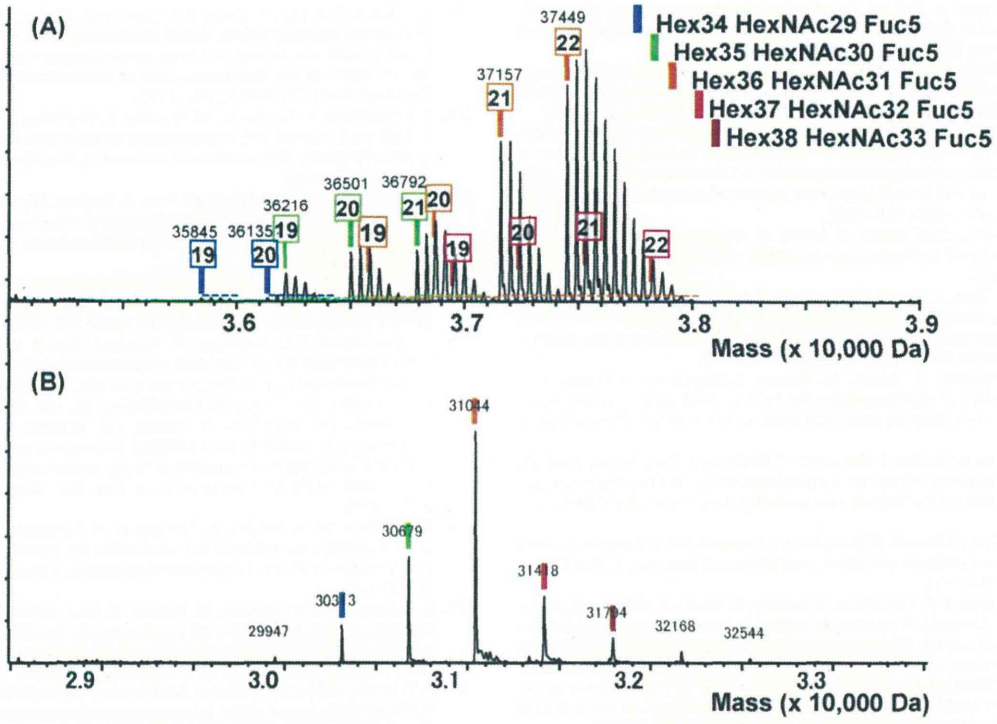

Fig. 5. Mass spectrometric glycoform profile of darbepoetin, an erythropoietin analog. (A) Total glycoform profile. (B) De-sialylated glycoform profile.

and biosimilar EPO products commercially available in Europe and Japan. Each product showed a characteristic pattern of glycoforms on the sialylation, asialo-glycan size, degrees of *O*-acetylation and addition of NeuGc for NeuAc or oxidation of the protein portion, and *O*-glycosylation. The most abundant number of sialic acid residues was 13, 12, 13, 12, 12, and 11 in EPO A, A′, B, C, C′ and C″, respectively. EPO A showed some *O*-acetylation in sialic acid residues. EPO A′ showed glycoforms with higher masses overall, indicating the presence of more LacNAc structures. EPO B showed higher sialylation, and the proportion of glycoforms with 14 sialic acid residues was higher than the others. EPO C showed a glycoform pattern similar to EPO A, but lower sialylation and higher *O*-acetylation. EPO C′ showed relatively higher sialylation in the total glycoforms and *O*-glycoform, and the proportion of glycoforms with 14 sialic acid residues was relatively high. EPO C′ also contained a small amount of glycoforms with high-mannose-type glycan containing M6P. There were very few glycoforms with 11 or less sialic acid residues, except for M6P-containing glycoforms that had 10 sialic acid residues. EPO C″ showed lower sialylation, and glycoforms having 14 sialic acid residues were very few. EPO C″ contained slightly more LacNAc structures. Although all the EPO products used in this study were produced using CHO cells, they showed a variety of glycoform profiles. All tested biosimilar products showed considerably different glycoform profiles from their innovator products. This indicates that it is very difficult to develop biosimilar products with similar glycan profiles as innovators in the case of highly heterogeneous biopharmaceuticals. In order to ensure the safety and efficacy of such products, it will be necessary to plan optimal strategies to evaluate the influences in the differences in the glycan profile on the safety and efficacy through pre-clinical tests, clinical trials and other means.

In the previous study, Taichrib et al. conducted glycoform profiling of EPO using CE-TOF MS [29]. They evaluate glycoform profiles using relative peak areas of selected intact erythropoietin isoforms and clearly distinguished commercial EPO products, preproduction preparations on the basis of the combined information on the antennarity, the sialoform, and the acetylation of the observed isoforms. Here, we performed glycoform characterization using the deconvoluted spectra derived from entire mass spectrum obtained by LC/ESI-MS. It is straightforward and easy to understand characteristics and patterns of glycoforms. Our data indicate that deconvoluted spectrum-based glycoform profiling is also available even for extremely complex glycoproteins. Mass spectrometric glycoform profiles showed a certain level of repeatability. Mass spectrometric glycoform profiling might be useful for the characterization of glycoform profiles during the development stage, evaluation of lot-to-lot glycoform heterogeneity, comparability between products before and after a change in the manufacturing process, the similarity between biosimilar products and their reference products, and monitoring of glycoform profiles during manufacturing processes.

Acknowledgement

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セミナー

一般名がわかるステムの知識

②生物薬品

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はじめに

本誌平成25年第1号では，化学薬品の一般名に使われるステムについて紹介するとともに，ステムが，化学構造，由来，薬理作用や効能・効果，及び標的とする生体分子などに基づき定義されていることを解説した。本号では，生物薬品（生体由来高分子医薬品，遺伝子組換え医薬品など）の一般名に使われるステムについて紹介する。生物薬品は，細胞・体液等から精製，もしくは遺伝子組換え技術により製造したペプチドや（糖）タンパク質等であり，生体内分子そのもの，もしくは生体内分子に類似した構造及び作用機序を持つ医薬品である。生物薬品のステムは，生体内分子の学術名の一部を用いて作られていることが多いが，それぞれのステムは，化学構造，由来，薬理作用や効能・効果，及び標的とする生体分子すべてを考慮して設定されているともいえる。例えば，エリスロポエチン類のステムは「ポエチン（poetin）」であり，poetinを持つ医薬品は，エリスロポエチンに類似したアミノ酸配列と赤血球分化増殖作用を持ち，エリスロポエチン受容体を標的とする医薬品と考えることができる。なお，遺伝子組換えで産生された医薬品には，エポエチン　アルファ（遺伝子組換え）のように，括弧書きで遺伝子組換えであることを加えるきまりがあり，生体由来品か遺伝子組換え品かを知ることができる。以下，本誌では（遺伝子組換え）を省略して，代表例を紹介する。

Key words　一般名／ステム／INN／JAN

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造血系，及び血液凝固系に由来する医薬品

前述したように，エリスロポエチン類のステムは「ポエチン（poetin）」である。わが国では，腎性貧血治療薬「エポエチン　アルファ（Epoetin Alfa）」，「エポエチン　ベータ（Epoetin Beta）」，「エポエチン　ベータ　ペゴル（Epoetin Beta Pegol）」，及び「ダルベポエチン　アルファ（Darbepoetin Alfa）」が承認されている。エポエチンとダルベポエチンにおける接頭辞の違いは，アミノ酸配列が異なることを示している。ステムの後に続くアルファやベータは，結合している糖鎖の違いを表している。ペゴルは，ポリエチレングリコール類（PEG）で修飾されていることを示している。尚，PEG化を表す方法として，他に，「ペグ（peg）」を接頭辞として用いる方法がある（次頁「免疫調整薬」のインターフェロン類参照）。

血液凝固因子のステムは「コグ（cog）」である。血液凝固第Ⅶ，第Ⅷ，及び第Ⅸ因子にはそれぞれサブステム「エプタコグ（eptacog）」，「オクトコグ（octocog）」，及び「ノナコグ（nonacog）」が決められている。活性型には括弧書きで「活性型（activated）」が付与される。代表的医薬品としてそれぞれ，「エプタコグ　アルファ（活性型）（Eptacog Alfa（activated））」，「オクトコグ　アルファ（Octocog Alfa）」，及び「ノナコグ　アルファ（Nonacog Alfa）」があり，血友病治療に用いられる。尚，人血液を原料とする医薬品の名称は生物学的製剤基準名であり，JANのルールには従わない。例えば，人血漿分画により製造された血液凝固第Ⅷ因子の名称は，乾燥濃縮人血液凝固第Ⅷ因子，または乾燥人血液凝固第Ⅷ因子である。

抗凝固作用をもつヘパリン類及びトロンボモジュリンには，ステムとしてそれぞれ「パリン（parin）」及び「トロンボモデュリン（Thrombomodulin）」が与えられている。代表的医薬品として，抗凝固薬「パル

ナパリン(Parnaparin)」及び汎発性血管内血液凝固症薬「トロンボモデュリン アルファ(Thrombomodulin Alfa)」がある。

免疫調整薬

コロニー刺激因子のステムは「スチム(stim)」である。顆粒球コロニー刺激因子には「グラスチム(grastim)」,及びマクロファージコロニー刺激因子には「モスチム(mostim)」のサブステムが決められている。グラスチムを持つ医薬品として,好中球減少症改善薬「フィルグラスチム(Filgrastim)」,「ナルトグラスチム(Nartograstim)」,及び「レノグラスチム(Lenograstim)」があり,いずれも第十六改正日本薬局方の第一追補から収載されている。巨核球系前駆細胞に直接作用し,血小板造血作用を持つ医薬品にもスチムが用いられる。慢性特発性血小板減少性紫斑病薬「ロミプロスチム(Romiplostim)」が承認されている。

インターフェロン(IFN)類のステムは「インターフェロン(interferon)」である。IFN-α,IFN-β,及びIFN-γはそれぞれ「インターフェロン アルファ(Interferon Alfa)」,「インターフェロン ベータ(Interferon Beta)」,及び「インターフェロン ガンマ(Interferon Gamma)」と2語式で表わされる。ただし,IFN類に限り,アルファやベータは糖鎖の違いを意味しているわけではない(前頁「造血系,及び血液凝固系に由来する医薬品」のエリスロポエチン類など参照)。アミノ酸配列が大きく異なるときは,アラビア数字を付与してインターフェロンアルファ-2のように表す。また,数個のアミノ酸残基の違いや糖鎖など翻訳後修飾が異なる場合は,小文字を追加して,インターフェロン アルファ-2aのように表す。さらに,日本独自のルールとして,括弧書きで培養細胞名を記載することがある。代表的な医薬品として,腎癌,C型肝炎薬「インターフェロン アルファ(NAMALBA)(Interferon Alfa(NAMALWA))」や「ペグインターフェロン アルファ-2b(Peginterferon Alfa-2b)」などがある。後者の接頭辞ペグはPEG化されていることを表している。

インターロイキン(IL)類のステムは「キン(kin)」であり,IL-1～4,6,8,10～13,18及び21にはそれぞれサブステムが与えられている。日本で承認されているIL類はIL-2類のみであり,IL-2のサブステム「ロイキン(leukin)」を用いて命名されている。抗悪性腫瘍薬として「セルモロイキン(Celmoleukin)」及び「テセロイキン(Teceleukin)」がある。

モノクローナル抗体のステムは「マブ(mab)」である。さらに,標的を示すサブステムAと種を示すサブステムBが決められている。心臓血管系,IL類,免疫系,骨,腫瘍,及びウイルスを標的とする抗体には,サブステムAとしてそれぞれ,「シ(c(i))」,「キン(kin)」,「リ(l(i))」,「ス(s(o))」,「ツ(t(u))」及び「ビ(v(i))」が用いられる。また,マウス抗体,キメラ抗体(マウスFabとヒトFcからなる抗体),ヒト化抗体(相補性決定領域がマウスに由来するヒト抗体),及びヒト抗体には,サブステムBとしてそれぞれ「オマブ(omab)」,「キシマブ(ximab)」,「ズマブ(zumab)」,及び「ウマブ(umab)」が与えられる。腫瘍を標的とするヒト抗体は日本語で「ツマブ(tumab)」と表記され,ヒト抗体であることが分かりにくくなっている。なお,サブステムAのルールは2009年に改正されているので,それ以前に一般名を取得した抗体医薬品の中には,このルールから多少はずれているものがある。抗体にキレート化剤,PEGまたは細胞傷害活性を有する薬物等が結合しているときは,抗体名の後ろに語を追加して二語式で表す。代表的な抗体医薬品に,抗悪性腫瘍薬「リツキシマブ(Rituximab)」,関節リウマチ治療薬「トシリズマブ(Tosilizumab)」,骨病変治療薬「デノスマブ(Denosumab)」,及び抗悪性腫瘍薬「ゲムツズマブ オゾガマイシン(Gemtuzumab Ozogamicin)」などがある。

受容体分子を含む医薬品のステムは「セプト(cept)」である。血管内皮増殖因子受容体,腫瘍壊死因子受容体,及び細胞傷害性Tリンパ球抗原4にはそれぞれ「ベルセプト(bercept)」,「ネルセプト(nercept)」及び「タセプト(tacept)」のサブステムが決められている。いずれも受容体の一部分とIgG1のFc領域が融合されたタンパク質である。我が国で承認されている医薬品として,加齢黄斑変性症薬「アフリベルセプト(Aflibercept)」,関節リウマチ薬「エタネルセプト(Etanercept)」及び関節リウマチ薬「アバタセプト(Abatacept)」がある。なお,セプトはFc受容体との融合タンパク質のステムではない。例えば,前述のロミプロスチムもヒトIgG1のFc領域とヒトトロンボポエチン受容体結合配列を含むペプチドとの融合タンパク質であるが,ステムはスチムである。

ホルモンに由来する医薬品

インスリン類のステムは「インスリン(insulin)」である。ただし,医薬品としての「インスリン(Insulin)」はブタもしくはウシ由来インスリンであり,ヒトインスリンと同じアミノ酸配列を持つ医薬品の名称は「ヒトインスリン(Insulin Human)」である。遺伝子組換え技術により製造されたインス

リンアナログは，インスリンの後にアミノ酸配列の違いを表す語を追加した2語式で表す。代表的なものに糖尿病治療薬「インスリン　リスプロ（Insulin Lispro）」や「インスリン　アルパルト（Insulin Aspart）」がある。

下垂体ホルモン類のステムとして，成長ホルモンには接頭辞として「ソム（som）」，卵胞刺激ホルモンのステムには「フォリトロピン（follitropin）」，副腎皮質刺激ホルモン類のステムには「アクチド（actide）」，オキシトシン類のステムには「トシン（tocin）」，及びバソプレシン類のステムには「プレシン（presin）」が定められている。成長ホルモン拮抗薬には接中辞として「ソム（som）」を用いる。代表的医薬品として，それぞれ，低身長症治療薬「ソマトロピン（Somatropin）」，排卵誘発薬「フォリトロピン　ベータ（Follitropin Beta）」，副腎皮質機能検査薬「テトラコサクチド（Tetracosactide）」，子宮収縮誘発薬「オキシトシン（Oxytocin）」，下垂体性尿崩症治療薬「バソプレシン（Vasopressin）」及び先端巨大症治療薬「ペグビソマント（Pegvisomant）」がある。胎盤由来の糖タンパク質性ホルモンには「ゴナドトロピン（Gonadotrop(h)in）」を用いる。無排卵症に用いられているヒト絨毛性性腺刺激ホルモン（Human Chorionic Gonadotrophin）」が該当する。

ホルモン放出刺激因子類のステムは「レリン（relin）」であり，サブステムとして，黄体形成ホルモン放出ホルモンには「レリン（relin）」，成長ホルモン放出促進ペプチド類には「モレリン（morelin）」，及び甲状腺刺激ホルモン放出ホルモン類には「チレリン（tirelin）」が用いられる。代表的医薬品として，それぞれ下垂体黄体形成ホルモン分泌機能検査薬「ゴナドレリン（Gonadorelin）」，成長ホルモン分泌不全症の診断薬「プラルモレリン（Pralmorelin）」及び脊髄小脳変性症における運動失調改善薬「タルチレリン（Taltirelin）」などがある。

ホルモン放出抑制ペプチド類には「レリクス（relix）」が用いられる。早期排卵防止薬「セトロレリクス（Cetrorelix）」，及び前立腺癌治療薬「デガレリクス（Degarelix）」などが承認されている。

その他に由来する医薬品

酵素類のステムは「アーゼ（ase）」である。プロティナーゼ，組織プラスミノゲン活性化因子，及びウロキナーゼ型プラスミノゲン活性化因子にはそれぞれサブステム「アーゼ（ase）」，「テプラーゼ（teplase）」，及び「ウプラーゼ（uplase）」が決められている。リソソーム酵素や糖分解酵素もアーゼを用いて命名される。代表的な医薬品に高血圧症治療薬「カリジノゲナーゼ（Kallidinogenase）」，血栓溶解薬「アルテプラーゼ（Alteplase）」，及びゴーシェ病に適応されている「イミグルセラーゼ（Imiglucerase）」などがある。

成長因子類のステムは「エルミン（ermin）」であり，線維芽細胞増殖因子のサブステムとして「フェルミン（fermin）」が決められている。日本では皮膚潰瘍薬「トラフルミン（Trafermin）」が承認されている。

以上に属さないペプチド及び糖ペプチド医薬品の命名には「チド（tide）」が用いられる。代表的医薬品として，2型糖尿病治療薬「エキセナチド（Exenatide）」や急性心不全薬「カリペリチド（Carperitide）」などがある。

バイオ後続品

バイオ後続品の名称は，先行バイオ医薬品の一般的名称（遺伝子組換えに係る記載を除く）の末尾に［後続1（2，3…）］を括弧書きで追加して表される。例えば，腎性貧血治療薬エポエチン　アルファのバイオ後続品は，「エポエチン カッパ（遺伝子組換え）［エポエチン アルファ後続1］（Epoetin Kappa (Genetical Recombination) [Epoetin Alfa Biosimilar 1]）」と表される。尚，成長ホルモンやインスリンのように，糖鎖などの修飾構造を持たないタンパク質（単純タンパク質）を有効成分とする場合は，新たに一般的名称を付すことなく，先行バイオ医薬品の一般的名称を用いる。ソマトロピン及びフィルグラスチムの後続品の名称はそれぞれ「ソマトロピン（Somatropin）」及び「フィルグラスチム（Filgrastim）」である。

最後に，日本で承認されている生物薬品で使われている主なステムとその定義（原則として，WHOのINN専門家協議の定義による）を表にまとめた。

表　生物薬品に使われる主なステムとその定義

	ステム		基原
	英語綴	日本語読み	
1	-actide	アクチド	副腎皮質刺激ホルモン類
2	-ase	アーゼ	酵素
3	-cept	セプト	受容体
4	-cog	コグ	血液凝固因子
5	-ermin	エルミン	成長因子
6	(-) follitropin	ホ/フォリトロピン	卵胞刺激ホルモン
7	-gonadotrop (h) in	ゴナドトロピン	絨毛性性腺刺激ホルモン
8	insulin	インスリン	インスリン類
9	interferon	インターフェロン	インターフェロン類
10	-kin	キン	インターロイキン類及び拮抗薬
11	-mab	マブ	モノクローナル抗体
12	-parin	パリン	ヘパリン(類)
13	-poetin	ポエチン	エリスロポエチン類
14	-pressin	プレシン	バソプレシン類
15	-relin	レリン	下垂体ホルモン放出刺激因子類
16	-relix	レリクス	下垂体ホルモン放出抑制ペプチド類
17	som-	ソム	成長ホルモン類及び拮抗薬
18	-stim	スチム	コロニー刺激因子類
19	thorombomodulin	トロンボモデュリン	トロンボモジュリン
20	-tide	チド	下垂体，視床下部，及び膵臓ホルモン以外のペプチド
21	-tocin	トシン	オキシトシン類

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3）International Nonproprietary Names (INN) for biological and biotechnological substances (a review) http://www.who.int/medicines/services/inn/BioRev2011.pdf

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ORIGINAL PAPER

Species identification of Asini Corii Collas (donkey glue) by PCR amplification of cytochrome *b* gene

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Abstract Asini Corii Collas (ACC; donkey glue) is a crude drug used to promote hematopoiesis and arrest bleeding. Because adulteration of the drug with substances from other animals such as horses, cattle, and pigs has been found, we examined PCR methods based on the sequence of the cytochrome *b* gene for source species identification. Two strategies for extracting DNA from ACC were compared, and the ion-exchange resin procedure was revealed to be more suitable than the silica-based one. Using DNA extracted from ACC by the ion-exchange resin procedure, PCR methods for species-specific detection of donkey, horse, cattle, and pig substances were established. When these species-specific PCR methods were applied to ACC, amplicons were obtained only by the donkey-specific PCR. Cattle-specific PCR detected as little as 0.1 % admixture of cattle glue in the ACC. These results suggest that the species-specific PCR methods established in this study would be useful for simple and easy detection of adulteration of ACC.

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Keywords Asini Corii Collas · Donkey glue · Species identification · Cytochrome *b*

Introduction

Asini Corii Collas (ACC; donkey glue) is a crude drug made of dry skin, bone, tendons, and ligaments of donkeys (*Equus asinus*) by decoction, degreasing, and concentration according to the Japanese standards for non-Pharmacopoeial crude drugs (non-JP crude drug standards) 2012 [1]. The main component is collagen. ACC has mainly been used in Kampo formulas such as Choreito and Unkeito in Japan, used for promoting hematopoiesis and arresting bleeding.

ACC is mainly produced in Shangton, China. In the Chinese Pharmacopoeia as well as in the non-JP crude drug standards, it is specified as being made of dry skin derived from donkeys [2]. However, adulteration with skins or bones from other animals such as horses, cattle, and pigs has been found [3, 4], because ACC is relatively expensive. Under these circumstances, the establishment of a method of discriminating the animal origin of ACC was needed. Several spectroscopy-based methods have been reported [5–7]; however, these methods have not reached the stage of practical application due to sample-processing variability.

Along with advances in DNA sequence technology, various methods for species identification of feed or processed food have been developed [8]. DNA-based authentication is one of the methods for identifying the animal source of ACC, but DNA extraction from ACC is considered to be difficult because its DNA is severely degraded during processing. In recent years, however, Lv et al. [3] succeeded in the extraction and detection of

severely degraded DNA from ACC. Furthermore, they reported a PCR method for authentication of ACC using SINEs (short interspersed nuclear elements) [4]. A SINE is a kind of retroposon, which is a short repetitive sequence with a length of about 300 bp, and has more than 10^6 total copies per haploid genome [9]. The method of donkey DNA detection using SINE consists of two PCR steps: the first PCR is based on the equine-specific ERE-1 region [10], and the second is based on the horse-specific satellite DNA region [11]. From donkey DNA, a PCR product should be generated by the first PCR, but not by the second. However, this method allows only indirect detection of donkey DNA, and cannot distinguish whether animal glues are derived from a mixture of donkey and horse or from 100 % horse. Although mitochondrial DNA has fewer copy numbers (several thousands) compared with those of SINE, it is widely used in species determination of animal meat and processed food; it has also been useful for assessing the evolutionary relationship between different species [12–14]. Furthermore, it can distinguish donkey from other animals including its close relatives, the horse [15]. In this study, we developed a more effective method of extracting DNA from ACC with reference to the method reported by Lv et al., and established a PCR method for source species identification of ACC based on the sequence differences of the cytochrome *b* gene in the mitochondrial DNA of several common mammals.

Materials and methods

Materials

ACC and cattle glue were purchased from Shandong Donge E-jiao Co. of Shandong Province.

A donkey hair sample was kindly provided by Hamura Zoo, Japan, with the aid of Dr. Sugita-Konishi. Meat samples from a horse, cow, and pig were purchased from local supermarkets or internet-based store. Their origins were identified by DNA analyses. One sample of each of these animal species was used in this study.

DNA extraction

Before DNA extraction, each side of solid glue samples was irradiated with UV light for 2 h in order to avoid a false-positive result due to the external DNA on the sample surfaces.

DNA extraction was done using a silica-membrane column (QIAquick spin column; Qiagen) according to the report of Lv et al. [3], and using an ion-exchange resin column (Qiagen Genomic tip 20/G or 100/G; Qiagen) with reference to the method for examining genetically modified foods (Ministry of Health, Labour and Welfare, Japan) [16].

For scale-up DNA preparation using the Genomic tip 100/G, 5 g solid glue was ground into powder and transferred into a 50-mL centrifuge tube with 16 mL of G2 buffer (Qiagen), 10 μL of RNase (100 mg/mL), and 200 μL of Proteinase K (20 mg/mL). After incubating the tube for 2 h at 50 °C with occasional shaking, the tube was centrifuged for 15 min at 6,000*g* at 4 °C.The supernatant was transferred into a 15-mL tube, and centrifuged again for 5 min at 10,000*g* at 4 °C.After equilibration of the Genomic tip 100/G with 4 mL of QBT buffer (Qiagen), the supernatant was applied to the tip and allowed to pass through the resin by gravity flow. The tip was then washed with 22 mL of QC buffer (Qiagen), and DNA was eluted with 5 mL of QF buffer prewarmed to 50 °C.The eluted DNA was precipitated by adding 3.5 mL of isopropanol, and centrifuged for 15 min at 10,000*g* at 4 °C. The pellet was washed with 5 mL of 70 % ethanol, and centrifuged. After drying the pellet at 65 °C, the pelleted DNA was suspended in 100 μL of distilled water, and stored at −20 °C until use.

DNA extraction from hair and meat samples was performed using Genomic tip 20/G according to the manufacturer's protocol.

For the comparison of DNA extraction methods, PCR was performed as reported by Lv et al. [3], using Ass-up/down primers.

Species-specific PCR

Multiple sequences of cytochrome *b* genes of donkeys, horses, cattle, and pigs were obtained from DDBJ/EMBL/Genbank, and the PCR primers listed in Table 1 were designed on the basis of the sequences specific to each animal species. A schematic of each primer is shown in Fig. S1. In designing the EaCytB-f primer, adenine (A), the fifth nucleotide from the 3′ end, was substituted for thymine (T) to enhance the specificity for donkey against the other three species [17].

PCR amplification was performed in a 25-μL reaction mixture containing 12.5 μL of Ampdirect Plus (Shimadzu), 0.75 U of Ex Taq Hot Start Version (Takara), 0.4 μM each of forward and reverse primer, and 5 ng (hair or meat samples) or 50 ng (glue sample) of template DNA. Amplification was conducted in a DNA Engine thermal cycler, PTC-200 (Bio-Rad) with the following conditions: 94 °C for 4 min; 35 cycles of 94 °C for 30 s, 60 °C (for donkey, horse, and cattle detection) or 64 °C (for pig detection) for 30 s, and 72 °C for 10 s; 72 °C for 2 min. PCR products were electrophoresed on a 4.5 % NuSieve GTG Agarose gel, stained with GelRed Nucleic Acid Gel Stain (Biotium). The amplicons were purified using