FcR カラムに直結した質量分析計による インタクト抗体の糖鎖解析

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Glycoform Analysis of Intact Antibodies Using FcR Column Online Mass Spectrometer

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Immunoglobulin G (IgG) is a widely used antibody that contains a glycosylation site in its Fc region. The type and combination of glycans vary greatly depending on the host in which the antibody is expressed. It has been reported that the glycostructure linked to the Fc-region strongly influences antibody-dependent cellular cytotoxicity (ADCC) activity, which is considered to be a risk factor when producing and developing antibody drugs.

We have created a technique for evaluating the glycan structure and ADCC activity associated with IgGs. It combines the separation of antibodies by an affinity analysis column (TSKgel® FcR-IIIA-NPR, Tosoh Corporation, Tokyo, Japan) with detection by mass spectrometry (MS). After analyzing commercially available monoclonal antibodies (mAbs) using this technique, we confirmed that affinity with the FcR column correlated with a defect in the core fucose and an increase in the amount of terminal galactose. Furthermore, it was applied to a commercially available antibody-drug conjugate and the same results as those for the monoclonal antibody were obtained.

抗体医薬品として広く利用されている免疫グロブリン（IgG）は、Fc 領域に糖鎖付加部位を含み、発現宿主によって付加する糖鎖の種類や組み合わせが大きく異なる。Fc 領域に付加した糖鎖構造が抗体依存性細胞傷害（ADCC）活性に大きく影響することが報告されており、抗体医薬品の製造・開発におけるリスク要因と考えられている。

我々は、東ソー株式会社が開発したアフィニティ分析カラム TSKgel FcR-IIIA-NPR による抗体の分離と質量分析計（MS）による検出を組み合わせることで、IgG に付加した状態の糖鎖構造と ADCC 活性を評価する技術を構築した。本技術を市販のモノクローナル抗体に適用し、コアフコースの欠損及び末端ガラクトース付加量の増加に相関した FcR カラムでの吸着の向上が確認された。さらに、抗体薬物複合体への適用を検討し、モノクローナル抗体と同様の結果を得ることができた。

1. Introduction

Recently, the research and development of antibody drugs that use antibodies (**Fig.1**) as an active ingredient

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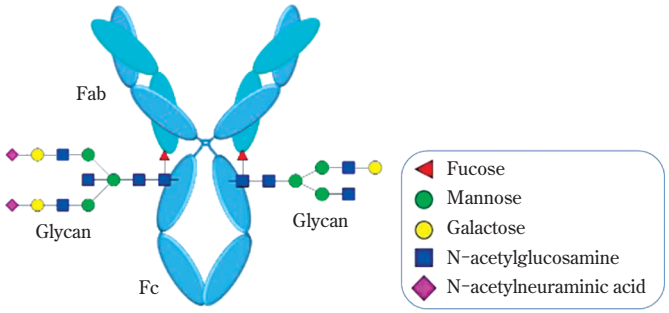


Fig. 1 Ganeral structure of antibodies

has been actively promoted. To date, more than 100 of these types of drugs have been approved worldwide, approximately 70% of which have been approved in Japan (as of March 2022)[1]. Immunoglobulin G (IgG), a widely used antibody drug, is a large glycoprotein with a bifurcated structure, has a molecular weight of approximately 150,000 Da, and is known to adopt diverse molecular structures as a result of various modification reactions during production[2]. Examples of post-translational modifications to IgG include heterogeneity of the C-terminal lysines, deamidation, oxidation, and glycosylation. Glycans linked to the Fc region (Fc glycans) have been reported to greatly affect antibody-dependent cellular cytotoxicity (ADCC) activity, which is considered a risk factor when producing and developing antibody drugs[3]. Antibodies that are produced mainly through cell culturing become heterogeneous mixtures with diverse glycan structures during the manufacturing to administration process, which means that a quality analysis of various components is required. However, monosaccharide monomolecules are fairly small with a molecular weight of about 180 Da, but antibodies can have a molecular weight of 150,000 Da. This means that advanced analysis techniques, such as complicated pretreatment and the use of expensive analysis equipment, are required when glycan structure and activity analyses of antibody drugs are carried out.

Therefore, the affinity analysis column TSKgel® FcR-IIIA-NPR (FcR column) for antibody drugs was developed by the Tosoh Corporation (Tokyo, Japan). The FcR columns recognize the Fc glycan, and because the antibody can be separated by the difference in its ADCC activity, it can more rapidly and conveniently analyze antibody samples than the conventional methods (LC-MS, MALDI-TOF/MS)[4].

In this article, we report a glycoform analysis using a mass spectrometer coupled directly to an FcR column (FcR-MS) as an applied technique that can be used to analyze antibody drugs. This technology contributes to quality control and the improvement of antibody drugs. The purpose of this technology is to contribute to the health components (achievement of a healthy and long-lived life) within the international targets set by the SDGs and to contribute to the goal of creating a foundation for industry and technology innovation in the form of advances in analytical technology.

2. Antibody glycans and ADCC activity

Immunoglobulin G contains a glycosylation site at residue Asn (residue 297) in the Fc region. It considerably affects the type and combination of glycans added by the expression host[5]. The glycan in the antibody is mainly composed of five monosaccharides: galactose (Gal), mannose (Man), fucose (Fuc), N-acetylglucosamine (GlcNAc), and N-acetylneuraminic acid (NeuNAc), which form diverse glycan structures through their complex links. Numerous reports have suggested that glycan structures and ADCC activity have important effects on antibody drugs. For example, core Fuc in the glycan structure reduces antibody binding to Fc receptor IIIa compared to IgG that lack Fuc, which reduces ADCC activity[6]. In contrast, increasing the amount of bisecting GlcNAc has been reported to enhance ADCC activity[7]. Moreover, IgG with terminal Gal exhibits a higher affinity for Fc-receptor IIIa because the terminal Gal enhances ADCC

activity[8].

The FcR column can analyze components based on the ADCC activity of an antibody. The column contains a chromatographic separation agent in which FcRγIIIa is fixed on the surface of a carrier, which means that the antibody molecules can be separated by differences in affinity[9]. When examining the relationship between the strong and weak ADCC activities derived from antibody separation by the FcR column and their glycan structure, the method used is to separate an antibody using an FcR column, followed by saccharide chain excision using enzymatic digestion or labeling and measurement by LC-MS. However, it is difficult to sufficiently confirm the association between separation by the FcR column and the Fc glycan because complicated pretreatment is necessary before the glycan is excised and measured using these methods. Therefore, the establishment of a new analytical method that can analyze the differences in the glycan structure of the antibody separated by the FcR column is desirable.

3. FcR-MS analysis

LC-MS analysis of intact proteins (intact protein analysis) is widely used to characterize proteins[10]. Intact protein analysis is a technique that is used to evaluate the molecular weight of a protein by subjecting a protein molecule to mass spectrometry in its original state without protease treatment. Therefore, it is possible to confirm the molecular weight in a state where a glycan is bound to an antibody and evaluate a complex where two glycans are bound to one antibody molecule.

In this study, we developed a technique capable of analyzing the glycoforms of FcR-separated antibodies by performing an intact protein analysis of antibodies using FcR-MS (**Fig.2**).

[1] Determination of the liquid chromatography conditions

The elution conditions for the antibodies when using an FcR column were examined. In FcR columns, many antibodies adsorb to FcRγIIIa when the pH is close to neutral (pH 6.0–7.5) and elute under acidic conditions (pH 4.0–5.0). Therefore, pH gradient elution with two solutions of varying pH is usually used. Citrate buffers with a wide buffering capacity in the above pH range are suitable eluents, but the use of nonvolatile salts in LC-MS reduces the volatility of the solvent, resulting in poor ionization during electrospray ionization (ESI), deposition on the ion source, and leads to clogging of the capillary. In this study, ammonium acetate was investigated as a volatile buffer in the required pH range. After considering the introduction of the sample into the MS equipment and the buffer range of ammonium acetate, gradient conditions based on two solutions of 20 mM ammonium acetate, pH 6.0 and pH 4.0, were set. The sample was UV-detected (280 nm) at a flow rate of 1.0 and 0.2 mL/min using 50 μg trastuzumab (Herceptin, Daiichi Sankyo, Tokyo, Japan) (**Figs.3**a, b). In addition, analysis in citrate buffer (pH 6.5 and pH 4.5) was performed for comparison (**Fig.3**c). Similar peaks to those for the citrate buffer were obtained when ammonium acetate was used, but there was a decrease in peak resolution compared to the citrate buffer.

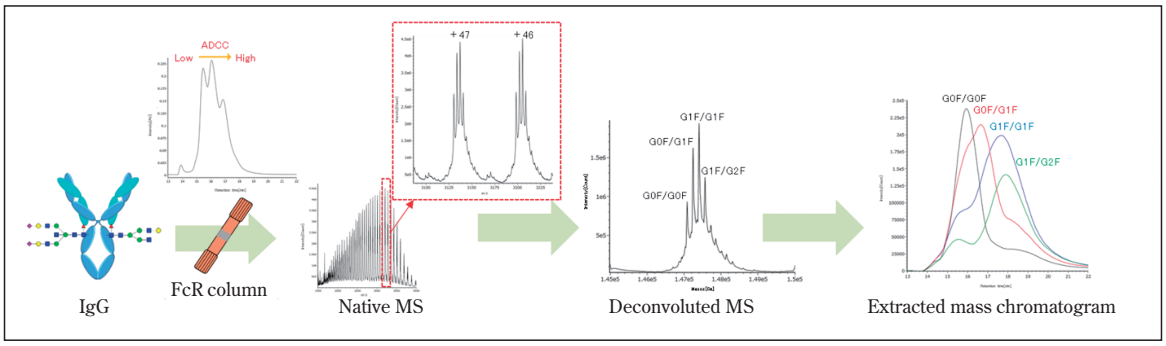
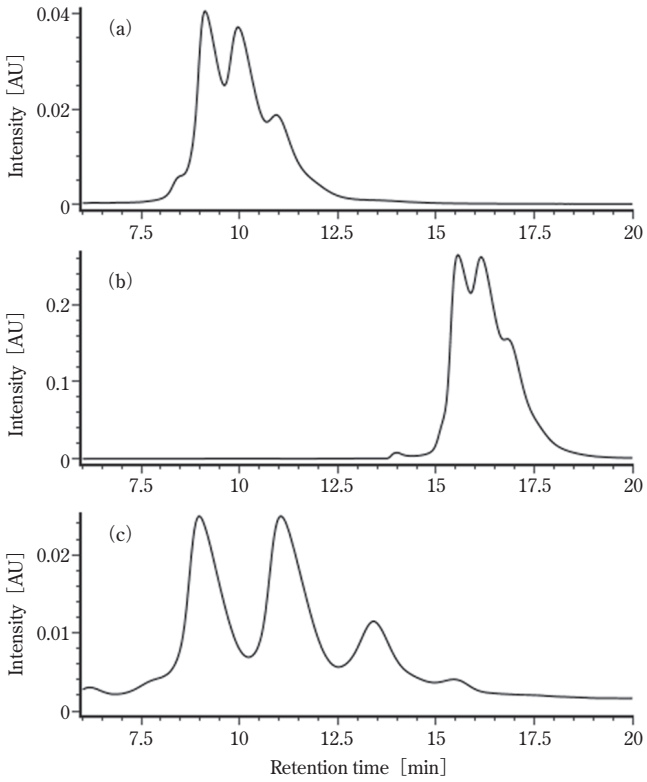


Fig. 2　Workflow of the FcR-MS analysis



(a) Flow rate 1.0 mL/ min with ammonium acetate,
(b) Flow rate 0.2 mL/ min with ammonium acetate,
(c) Flow rate 1.0 mL/ min with sodium citrate

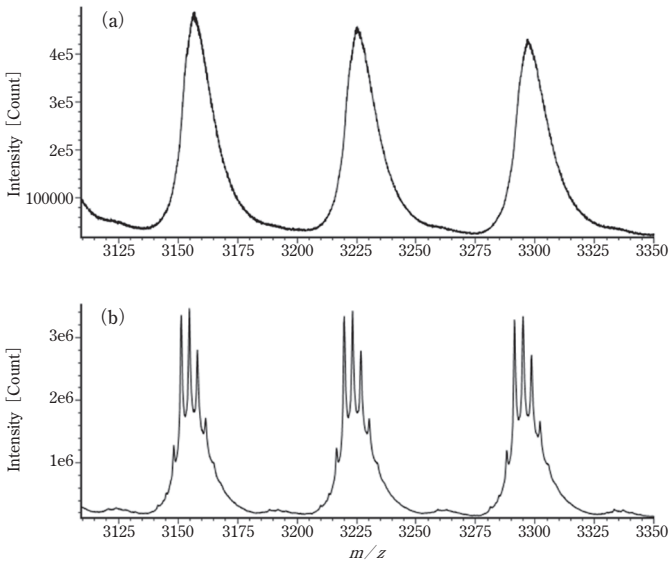
Fig. 3 Chromatographic profile of trastuzumab using the FcR column

[2] Investigation of MS measurement conditions

The LC-MS measurement conditions were then examined. Samples were eluted with 50 μg of trastuzumab using an ACQUITY UPLC H-class bio (Waters, Milford, MA, USA) at a flow rate of 0.2 mL/min, followed by mass spectrometry. The mass spectrometer was measured in the positive ion mode using a Xevo G2-XS QTof (Waters). Mass spectrometry performed after ammonium acetate elution revealed that the mass spectra became smooth, indicating low resolution. Therefore, confirming the difference between Fc glycans was difficult because they could not be separated (**Fig.4**a). This was presumed to be due to the decrease in sensitivity caused by the low volatility of the aqueous solvent and a decrease in mass resolution due to competition by the ammonium addition ion for the proton addition ion to be detected during ionization in at the ESI stage. To solve these problems, we examined whether sensitivity could be improved by adding an organic solvent and formic acid in a post-column using a makeup pump. To ensure that the solvents were thoroughly mixed together, the flow path was connected using a non-metal static mixer (GL Science, Tokyo, Japan). When 50% acetonitrile containing 1.0% formic acid was used as an additional solvent and added at a volume of 50 v/v% with the LC eluate after separation by the FcR column, the resolution of the MS spectrum improved and separation of the spectrum due to the differences in the Fc glycans was confirmed (**Fig.4**b). This improvement was presumed to be due to the fact that the addition of an organic solvent decreases the surface tension of the eluate and improves volatility. Moreover, the addition of formic acid increases the supply of protons, thereby alleviating the influence of ammonium ions.

[3] Examination of analysis conditions

We used the mass spectrum acquired in Section 3 [2] and shown in **Fig.4**b to perform a deconvolution analysis. The protein characterization software UNIFI (Waters) attached to the device was used for the analysis. The antibody masses calculated from the published amino acid sequence information in Drug Bank[11] were used, and an analysis was performed to



（a）Ammonium acetate only,
（b）Addition of post-column solvents（1.0% FA in 50% ACN）

Fig. 4　Mass spectrum of the intact trastuzumab

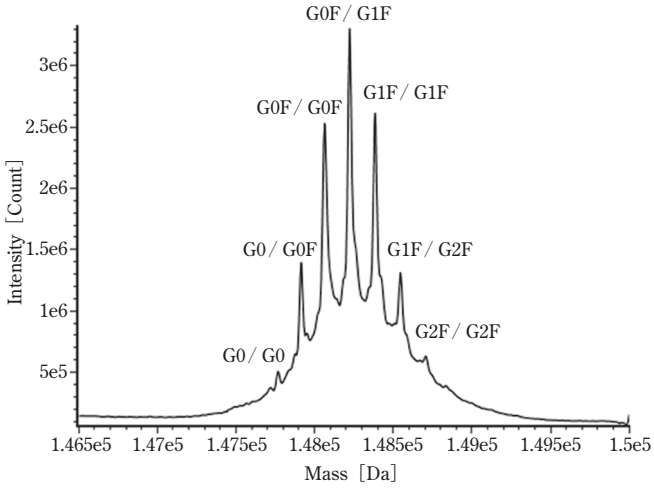


Fig. 5　Deconvoluted mass spectrum of trastuzumab

search for glycans (Man5, G0, G0F, G1F, and G2F) present at levels of 1% or more using an excised glycan analysis, which was performed in advance (**Fig.5**). The glycan names and structures used in this study are listed in **Table 1**.

The mass-to-charge ratio (m/z) in the polyvalent ion was calculated from the mass of each linked glycan obtained from the analysis results and a chromatogram for each extracted ion was generated. These were overlaid to generate a chromatogram (FcR-MS chromatogram) reflecting the glycan conformation of the FcR-column-separated antibody (**Fig.6**). In the FcR-MS chromatogram, G0F/G0F containing core Fuc, G0/G0F without Fuc, and G0/G0 without Fuc were eluted in order and the results indicated an improvement in ADCC activity due to the lack of core Fuc. Furthermore, the dissolution slowed with each increase in terminal Gal addition, which also supports a previous report[12].

4. Case Study

To confirm the versatility of the novel FcR-MS analysis technique used in this study, we analyzed cases involving monoclonal antibodies (mAbs) and antibody-

Table 1 Glycostructures commonly found in mAbs

Name and composition	Classification	Structure
Man5 [H5N2]	High mannose	
G0 [H3N4]	Complex, nonfucosylated	
G0F [H3N4F1]	Complex, fucosylated	
G1F [H4N4F1]	Complex, fucosylated	
G2F [H5N4F1]	Complex, fucosylated	

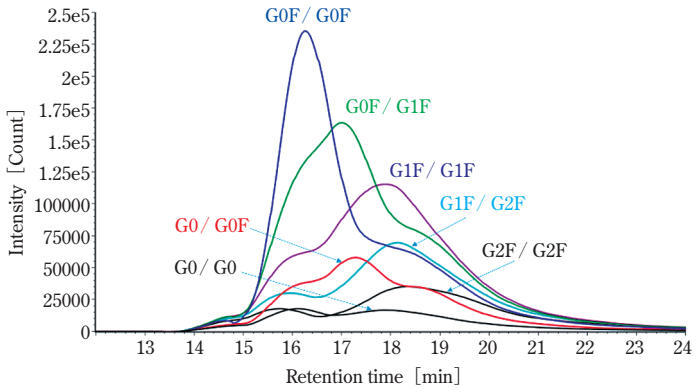


Fig. 6 Extracted mass chromatograms of trastuzumab

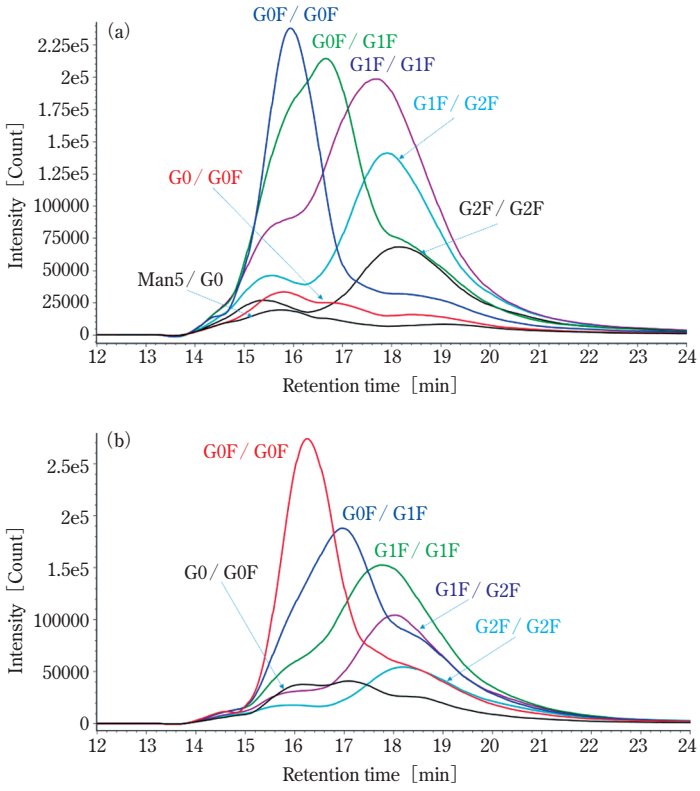
drug conjugates (ADCs).

[1] Monoclonal antibody

The samples contained 50 μg each of rituximab (Rituxan, Zenyaku Kogyo, Tokyo, Japan) and tocilizumab (Actemra, Chugai Pharmaceutical, Tokyo, Japan). The analyses were performed under the same conditions described in Section 3. The mAbs masses were calculated using the published amino acid sequence information in the Drug Bank[11] and then the analyses were performed (**Fig.7**). Similar to trastuzumab, enhanced ADCC activity due to Fuc deficiency and increased terminal Gal levels were confirmed, which showed that this method is applicable to common mAbs.

[2] Antibody-drug conjugates

An antibody-drug conjugate (ADC) is a monoclonal antibody covalently conjugated (complexed) to a small-molecule drug using an ADC linker[13]. The samples were measured and analyzed using trastuzumab emtansine (Kadcyla, Chugai Pharmaceutical) under the same conditions described in Section 3 [1] (**Fig.8**). Trastuzumab emtansine had 1–10 molecules of



（a）Rituximab,（b）Tocilizumab

Fig. 7　Extracted mass chromatograms

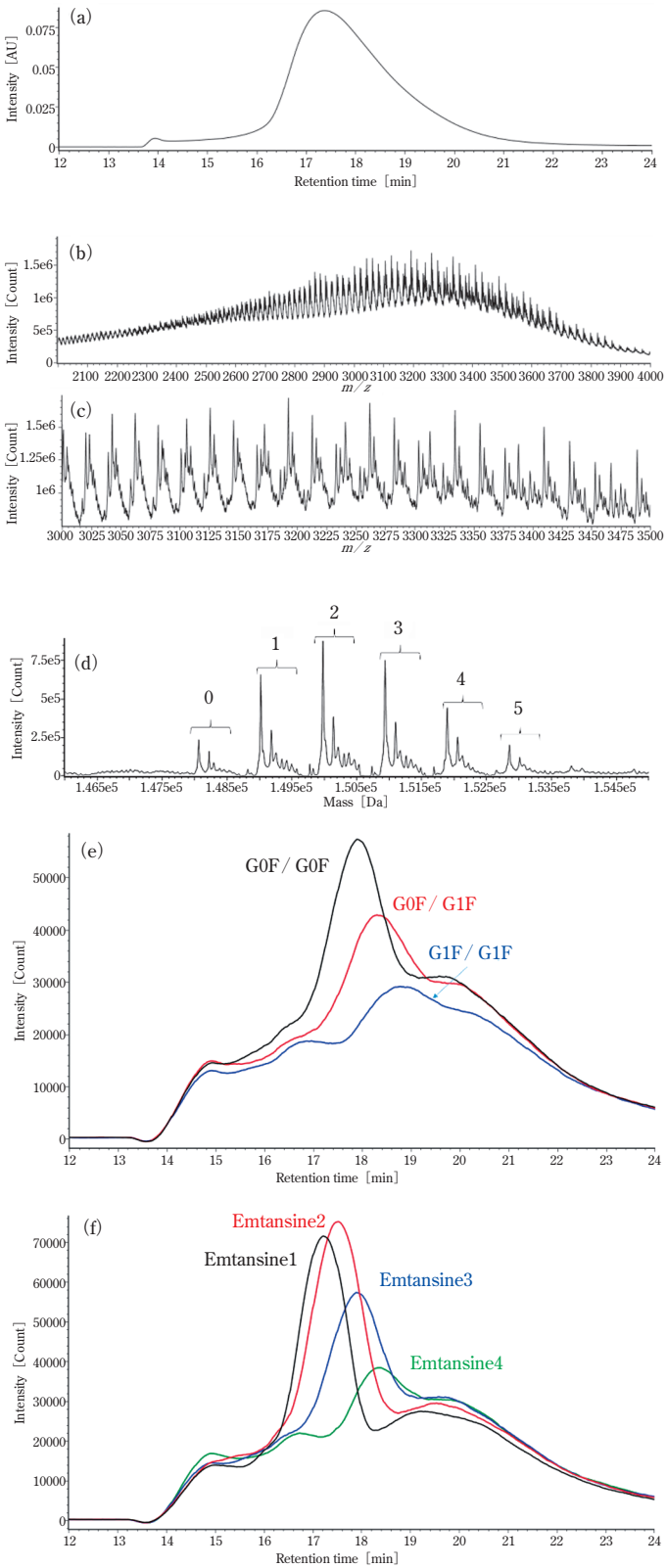
DM-1 bound to the amino groups of lysine residues because trastuzumab adds a mean of 3.5 cytotoxic emtansines (DM-1). The UV chromatogram shows a single smooth peak (**Fig.8**a) and the mass spectrum shows that neighboring multiply charged ions were detected compared to trastuzumab (**Figs.8**b, c), which suggests that variations in the additional numbers of DM-1 molecules can be detected. The deconvolution analysis detected trastuzumab with 0–5 additional DM-1 molecules (**Fig.8**d). Extraction chromatograms of the different glycan linkages (G0F/G0F, G0F/G1F, and G1F/G1F) in the antibody with two DM-1 additions showed that elution slowed with each terminal Gal addition, even when DM-1 was added. This indicates that the antibody ADCC activity was similar to trastuzumab (**Fig.8**e). Extraction chromatograms of antibodies with the same glycan linkage (G0F/G0F) but with different numbers of DM-1 additions showed that elution slowed as the number of DM-1 additions increased (**Fig.8**f). This is consistent with studies where trastuzumab and its antibody-drug complexes were measured in FcR-columns. They also showed that drug binding delayed the elution peak[14]. The ADCC mechanism of action in trastuzumab emtansine is thought to originate from the Fc region of trastuzumab. However, our findings suggest that antibody-bound DM-1 may improve the affinity of antibodies for FcRγ IIIa.

5. Summary

To investigate the relationship between the separation of antibodies on the FcR column and the glycan structure to be bound, a new analytical method was created that used a mass spectrometer coupled directly to an FcR column.

A novel FcR-MS analysis technique was created using a volatile buffer and by adding organic solvents and formic acid to the post-column. Then determinations of commercially available antibody drugs were undertaken.

This method can be used in the quality control of



(a) Chromatographic profile using the FcR column,
(b) Mass spectrum m/z 2000–4000,
(c) Mass spectrum m/z 3000–3500,
(d) Deconvoluted mass spectrum,
(e) Extracted mass chromatograms when two DM-1 are bound,
(f) Extracted mass chromatograms for G0F/G0F

Fig. 8 FcR-MS analysis of trastuzumab emtansine

antibody drugs, to optimize cell culture conditions, and in screening procedures for new drug development. It is also expected to be utilized in antibody drug production and development.

6. Acknowledgements

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