國立臺灣大學醫學院藥學研究所

博士論文

School of Pharmacy
College of Medicine
National Taiwan University
Doctoral Dissertation

以質譜法開發偵測癌症之生物標誌與定量人體血漿中之單株抗體藥物

Using mass spectrometry for investigating cancer detection markers and quantifying therapeutic monoclonal antibodies in human plasma

邱懷萱

Huai-Hsuan Chiu

指導教授：郭錦樺 博士

Advisor: Ching-Hua Kuo, Ph.D.

中華民國 107 年 10 月

October, 2018

doi:10.6342/NTU201804105

致謝

博士班的時光是一段奇妙的旅程，這一路上受到了許多人的提攜與幫助，我才能夠順利完成學業。感謝我的指導教授郭錦樺老師，在研究上總是不厭其煩的教導、鼓勵我，帶領我一起克服實驗的困難，在生活上關心、照顧我，能夠跟著老師學習，真的是一件非常幸福的事。

感謝口試委員李仁愛老師、賴建成老師、陳逸然老師及陳頌方老師，謝謝老師們詳細審查我的論文，在口試時給予許多寶貴的建議以及指正，讓我的論文內容能夠更加完整，並且知道自己在研究上還有哪些地方可以做的更嚴謹，著實獲益良多，也非常感謝口試委員們對我的鼓勵，讓我有信心、動力繼續往下一個目標邁進。

謝謝實驗室的學長姐們在研究上的幫助以及鼓勵。感謝伊琳學姊總是溫暖的關心我的生活，耐心的教我實驗；感謝冠元學長在儀器操作上的教學，常常和我討論許多實驗的想法；感謝松政、崴清在數據分析統計上的支援；感謝淑涵一直幫我加油打氣；感謝 Marisa 在校稿上的協助，能夠成為你們的同學，一起學習、成長，真的很幸運；謝謝學弟妹們帶給我許多歡笑以及實驗室助理們的各種支援，能夠與大家一起在實驗室學習真的很開心。

感謝我最愛的家人們，謝謝我的爸爸媽媽，永遠扮演我最堅強的後盾，您們的陪伴與疼愛，是讓我能夠堅持下去的動力。謝謝我的弟弟，能和你一起念同一個研究所，一起分享學校的點點滴滴，陪伴彼此度過無數個實驗失敗的低潮，真的是一個很難得又幸運的機緣，相信你一定也會很順利的完成你的學業。謝謝我的妹妹，有妳這個溫暖又貼心的妹妹，真的是一件很幸福的事情!謝謝妳在我沮喪難過時，陪我聊天，跟我分享笑話，逗我開心。謝謝曉偉在我面臨低潮時陪伴我，安撫我焦慮的心情，也在實驗上和我一起腦力激盪。謝謝疼愛我的阿姨對我的諸多照顧，總是在我需要幫忙時伸出援手。謝謝從小呵護我的阿嬤、外公、外婆，我知道您們一定在天上保佑著我。

doi:10.6342/NTU201804105

感謝這一路上陪伴我、支持我的師長、家人與朋友們，希望未來，我也能成為一個可以給予別人幫助、有所貢獻的人。僅以這篇博士論文，獻給我最愛的家人與所有幫助過我的人。

doi:10.6342/NTU201804105

中文摘要

癌症一直是全球人類死亡的主要原因之一，許多的研究，例如癌症風險因子評估、癌症的偵測方法、以及改善癌症的治療都是為減少其致死率。透過生物標誌的尋找，可以幫助病人早期發現疾病，達到早期診斷早期治療的效果。此外，由於單株抗體類的藥物常被廣泛的應用在癌症的治療上，而目前許多單株抗體藥物類的藥物動力學與藥效學還在進行研究，因此提供一個準確、精確的定量方法將有助於這些研究的進行。本研究使用氣相層析質譜儀與液相層析質譜儀來開發生醫分析方法應用於癌症偵測以及單株抗體藥物治療，期望可以改善癌症的治療，達到促進個人化醫療的契機。

本論文分成兩個部分，第一部分我們使用標的代謝體學方法在氣相層析質譜儀平台上尋找具有潛力的脂肪酸做為乳癌的生物標誌。早期偵測乳癌不但可以減少致死率，同時也可以提升治癒率。為了尋找具有潛力的脂肪酸作為生物標誌，我們利用氣相層析質譜儀開發代謝體學的脂肪酸分析方法，測定血漿樣品中的脂肪酸組成。血漿樣品經由乙醯氯進行衍生化反應，以氣相層析質譜儀進行分析，而確效的結果也顯示大於 90%的定量結果誤差小於 15%，在日內以及異日間的再現性評估，超過 90%的結果其相對標準偏差都落在 15%以內。我們進一步分析了健康受試者以及乳癌病患的血液中脂肪酸的含量。考量停經可能會影響脂肪酸的組成，我們將樣品分為停經前後的組別。本研究觀察到在停經後組別中，乳癌病患的血漿樣品內，C18:2n6、C22:0、C24:0 這三個脂肪酸

doi:10.6342/NTU201804105

的含量顯著低於健康受試者，而在停經前的組別中，除了上述三個脂肪酸外，還有 C16:0、C18:0、C18:1n9c、C20:0、C20:4n6、C22:6n3 以及 C24:1n9 在乳癌病患血漿樣品中的含量也都顯著低於健康受試者。我們也進一步利用這些具有潛力的乳癌生物標誌建立乳癌的預測模型。最佳化後的預測模型如下: 在停經後以 C18:2n6、C22:0、C24:0 建立的預測模型，其受試者操作特徵曲線下部分面積數值為 0.72，靈敏度與專一性數值分別為 70.6%以及 70.4%。而在停經前的組別當中，我們分別以 C22:0、C24:1n9 建立的預測模型，其受試者操作特徵曲線下部分面積數值為 0.78，靈敏度與專一性數值分別為 73.3%以及 70.8%。

第二部分使用液相層析質譜儀開發兩個單株抗體藥物的血中濃度分析方法，我們先建立了一個以 Protein G 磁珠樣品前處理的方法，從複雜的血液樣品基質當中，純化目標分析物，單株抗體藥物－Bevacizumab，並利用另一個 immunoglobulin G (IgG)類的單株抗體藥物－Tocilizumab 做為內標，校正在樣品配置過程中，可能產生之誤差。分析方法確效後，我們將本方法應用於實際臨床檢體之測量，定量病患體內的 Bevacizumab 濃度。結果顯示，儘管給予的 Bevacizumab 劑量相同，採血的時間點也相同，病人個體之間的藥物血中濃度仍然會具有達到兩倍多的差異。本方法可以應用於 Bevacizumab 的藥物動力學以及藥效學的研究，並提供一個監測 Bevacizumab 藥物血中濃度的方法。

有鑑於免疫球蛋白 G (IgG) 類的藥物在目前大部分經由美國食品藥物管

doi:10.6342/NTU201804105

理局核准的單株抗體物中佔有很高的比例，為了促進這類型藥物的藥物動力學以及藥效學的研究，並提供藥物濃度監測的方法，我們另外開發了一個具有廣用性的液相層析質譜儀分析平台，定量血液樣品中 IgG 類的單株抗體藥物濃度，並以三個 IgG 類的藥物:Pembrolizumab、Nivolumab、Bevacizumab 做為測試藥物，來證明平台的應用性。我們選擇了 protein G 作為我們的樣品純化方法，並發展雙內標校正法，使用一個 IgG 類的藥物 tocilizumab 做為樣品配置時的內標，搭配柱後注入內部標準品方法。研究結果顯示，三個測試單株抗體藥物的定量準確度的數值都落在 100± 15%之間，在日內以及日間的再現性評估，相對標準偏差也都落在 15%以內，顯示了雙內標校正法可以有效改善定量準確度。我們將分析方法應用於臨床血液檢體的波谷濃度點，定量結果顯示本方法具有足夠的靈敏度可以定量三個測試藥物的血中濃度。

本論文透過標的代謝體學的方法，尋找具有潛力的脂肪酸做為乳癌生物標誌，並開發單株抗體藥物血中濃度的測量方法。這些方法都經過分析方法確效，並且實際應用在臨床檢體的分析以確認方法的應用性。未來也期望這些分析方法可以更廣泛的使用於臨床，達到個人化醫療的最終目標。

關鍵字：癌症、生醫分析、代謝體學、氣相層析質譜法、生物標誌、液相層析質譜法、單株抗體藥物、Protein G 純化、柱後注入內部標準品校正方法

doi:10.6342/NTU201804105

Abstract

Cancer is one of the leading causes of death globally. To fight this disease, the researches such as the risk factors of cancer, cancer detection methods, and cancer treatment are all growing rapidly in recent years. Biomarker discovery was one of the potential strategies for early detection of cancer. In addition, due to the promising efficacy of therapeutic monoclonal antibodies (mAbs), these types of drugs are frequently used in the cancer treatment. Many studies are still investigating the pharmacokinetic (PK) and pharmacodynamic (PD) properties of therapeutic mAbs. Therefore, an accurate and precise quantification method would facilitate these studies. This study used both gas-chromatography mass spectrometry (GC-MS) and liquid-chromatography mass spectrometry (LC-MS) platforms to develop bio-analytical methods for cancer detection and quantification of therapeutic monoclonal antibodies (mAbs) drugs, and hopefully to improve cancer treatment and also to facilitate precision medicine.

This thesis is divided into two parts. In the first part, we used a targeted metabolomics approach to investigate the potential fatty acids as the biomarkers for breast cancer (BC) detection on GC-MS. Detect BC at early stage could not only reduce the cancer mortality but also improve the treatment outcome. To investigate potential

doi:10.6342/NTU201804105

fatty acid biomarkers, we developed a fatty acid profiling method for plasma metabolomic studies by GC-MS. The plasma samples were derivatized by acetyl chloride, and then analyzed by GC-MS. This analytical method was validated with accuracy within 100 ± 15%, and the intraday and interday precision were lower than 15% RSD for over 90% analytes. We further analyzed fatty acid profiles in both healthy volunteers and BC patients. To consider that the fatty acid profiles would be affected by the menopausal status, we divided the samples into pre- menopausal and post-menopausal. In post-menopausal group, the concentrations of three fatty acids including C22:0, C24:0, C18:2n6 were significantly lower in BC patients. In addition to C22:0, C24:0, C18:2n6, seven fatty acids, including C16:0, C18:0, C18:1n9c, C20:0, C20:4n6, C22:6n3, and C24:1n9, showed significant differences in pre-menopausal patients. We further applied these potential BC biomarkers to build the prediction model. The optimal prediction model for post-menopausal group was built by C18:2n6, C22:0 and C24:0 with the area under the curve (AUC) value of 0.72 (70.6% sensitivity and 70.4% specificity). In pre-menopausal group, the prediction model was built by C22:0 and C24:1n9, with the AUC value of 0.78 (73.3% sensitivity and 70.8% specificity). In the second part of this thesis, we developed two analytical methods for therapeutic mAb quantification in human plasma samples. In the first study, we developed a protein G purification method to trap the target analyte, bevacizumab, from human plasma, and

doi:10.6342/NTU201804105

applied another immunoglobulin G (IgG) based drug tocilizumab as the internal standard to correct the potential loss during sample preparation procedure. The method was validated, and then used to quantify the bevacizumab concentrations in patients' plasma samples. The quantification results revealed that bevacizumab concentrations fluctuated significantly between individuals even though the dosage of bevacizumab and the collection time point were both the same for the tested individuals. This method could be applied to investigate the PK and PD properties of bevacizumab, and also for conducting therapeutic drug monitoring (TDM) of bevacizumab.

IgG represents a high percentage of mAb drugs that have been approved by the Food and Drug Administration (FDA). To facilitate therapeutic drug monitoring and PK/PD studies, we developed a general LC-MS/MS method to quantify the concentration of IgG-based mAbs in human plasma. Three IgG-based drugs (bevacizumab, nivolumab and pembrolizumab) were selected to demonstrate our method. Protein G beads were used for sample pretreatment, combined with a two internal standard (IS) calibration method that includes the IgG-based drug-IS tocilizumab and post-column infused IS. The results indicated that the accuracy for three demonstration drugs were all within 100 ± 15%. The intraday and interday precision were lower than 15% RSD. Using two internal standards was found to effectively improve quantification accuracy. The successful application of the method

doi:10.6342/NTU201804105

to clinical samples at trough levels demonstrated its' applicability in clinical analysis

This thesis used a targeted metabolomic approach to investigate the potential fatty acids as the BC detection biomarkers, and developed LC-MS/MS methods for quantifying therapeutic mAbs concentrations in human plasma. All of these methods were validated, and applied to clinical samples to investigate the method applicability. It is anticipated that these methods could be applied to clinical practice, and to achieve the goals of personalized medicine.

Keywords: cancer, biomedical analysis, metabolomics, gas chromatography–mass spectrometry, biomarker, liquid chromatography- mass spectrometry, therapeutic monoclonal antibodies, protein G purification, post-column infused internal standard method

doi:10.6342/NTU201804105

Contents

doi:10.6342/NTU201804105

doi:10.6342/NTU201804105

doi:10.6342/NTU201804105

doi:10.6342/NTU201804105

Figure contents

doi:10.6342/NTU201804105

Table contents

doi:10.6342/NTU201804105

doi:10.6342/NTU201804105

Chapter 1. Introduction

doi:10.6342/NTU201804105

1.1 Cancer

Cancer is the name given by a collection of related diseases, which contains abnormal cells. The abnormal cells may invade or spread to other parts of the body, and may make cancer treatment become a difficult task. According to data from World Health Organization (WHO), cancer caused 8.8 million deaths in 2015, which was the second leading cause of death in the worldwide. Currently, nearly 1 in 6 deaths is due to cancer, which is a relatively high ratio. In addition, cancer is a high occurrence disease in the world, and affecting the life quality significantly. In order to fight with this common but serious disease, the researches about the risk factors of cancer, cancer detection method, and the improvement of cancer treatment are all growing rapidly in recent years.[1-10]

During last two decades, the cancer mortality in the United States, Europe, and other high-income countries was decreasing.[11-13] This is due to the improvement in medical science, such as early diagnosis and also various new drugs being available.[14] Early diagnosis could be achievable by several ways, and biomarker detection is one of the potential strategies.[15] Biomarker is defined as a measurable indicator in some biological status or conditions.[16] Biomarker discovery shows the potential to assist physicians to achieve early detection of diseases, and it is also gaining more importance for precision

doi:10.6342/NTU201804105

medicine.[17-18] Biomarker discovery for disease is no longer an unrealistic dream due to the development of “omic” sciences including proteomics, genomics, metabolomics and so on. These “omic” sciences provide the comprehensive information to discover biomarkers which may be benefit to both early diagnosis and treatment selection.

The other method to reduce the cancer mortality is to improve cancer treatment. The conventional methods for cancer treatment includes surgical therapy, radio therapy, and chemotherapy. Due to the significant advance in medical science over past 20 years, target therapy and immunotherapy are now also becoming treatment selections for cancer therapy. Therapeutic monoclonal antibodies (mAbs) were commonly applied to perform both target therapy and immunotherapy in recent years.[19-20] Compared to surgery, chemotherapy and radiotherapy, using mAb for cancer treatment can provide better specificity and lower toxicity, therefore, improving the treatment efficacy.

1.2 Using metabolomics for breast cancer detection

Breast cancer (BC) is the most common female cancer with high incident rate in worldwide.[21-22] There are 1.7 million new cases per year, and the percentage of breast cancer in all types of cancers is nearly 25% in female. It is estimated that around 0.5 million people died due to this disease.[22-23] In Taiwan, breast cancer incident rate is also

doi:10.6342/NTU201804105

very high among women, the incident rate has significantly increased since last two decades.

The survival rate of BC is strongly related to the disease status at diagnosis, the 10-year survival rate is around 98% for patients who were diagnosed at early stage (0 and I stage) compared with a 65% 10-year survival rate for patients with stage III disease.[24-26] In addition, the outcome of BC patient is significantly influenced by cancer stage at the time of diagnosis.[27] Therefore, it is important to have methods for early detection of BC. The current screening approaches for BC includes breast self-examinations, clinical breast examinations and breast imaging. The good accuracy, acceptable sensitivity and specificity, were all the requirements for a BC screening method. However, even mammography is currently the gold standard method for breast cancer screening, it still has some limitations such as higher false rate for women with dense breasts.[28-29] In addition, the increasement of risks due to repeated exposure to harmful ionizing radiations.

Recently, due to the development of science, the results acquired from the genomic testing, proteomic profiling and metabolomics provided additional information for selecting treatment approaches and managements.[30-32] Among these omic sciences, metabolomics is becoming a hot issue in recent years. It is firstly described by Nichoson *et al*. in 1993, with the definition as ”the quantitative measurement of the dynamic

doi:10.6342/NTU201804105

multiparametrix metabolic response of living system to pathophysiological stimuli or genetic modification."[33] Metabolomics research approaches can be briefly divided into targeted and non-targeted. The metabolomics study is mainly focus on small-molecule metabolites (< 1500 Daltons) in a biological specimen (such as plasma, urine), and these metabolites could reflect most closely to the phenotype of an organism at specific time and status. The concentration of these metabolites often varies during disease status compared to healthy conditions. As a result, metabolomic presents a promising tool to explore the potential disease biomarkers due to its' ability to measure these relative metabolites and correlate to the physiological status. Several metabolomic researches have identified the potential biomarkers for various disease, and also for breast cancer. [34-37] Measurement of the fluctuations of certain metabolites in bio-fluids is a promising strategy to detect breast cancer at early stages.[38-39]

In non-targeted metabolomic studies, relative quantification using raw signal intensity has been commonly applied as indicators of the metabolite level. This is due to that it is impractical to construct the calibration curves for all metabolites, and it is also difficult to select appropriate internal standards for correction all of the analytes. Therefore, it is really hard to perform precise and accurate quantification of all metabolites in bio-fluid in non-targeted studies.[40-41] However, to make a biomarker be applied from lab to the clinic, an accurate and precise method for metabolites

doi:10.6342/NTU201804105

quantification is a critical point.[42-43]

1.3 Cancer treatment with therapeutic monoclonal antibody (mAb)

Cancer treatment with different kinds of therapeutic mAbs has become one of the most successful therapeutic approaches since last decades.[44] MAb has become a hot issue in the medical field and pharamaceutical community and it has gained high attentions among the biotech drugs in development. Many mAbs are currently used for cancer therapies including breast cancer, colorectal cancer, lung cancer and so on. There are also many mAbs currently undergoing clinical evaluation, and these drugs generally show better specificity with lower side effects and better efficacy compared to conventional small molecule drugs due to their target-specific mechanisms. Although mAbs provide better safety profile, the clinical outcomes may vary among patients in some cases, and the extent of the side effects also varied.[45] In addition, the pharmacokinetic (PK) and pharmacodynamic (PD) properties of therapeutic mAbs were different to conventional small molecules and are being more complicated.[46-47] To investigate the PK/PD properties of mAbs and to improve the clinical outcomes for mAb therapy, an accurate method to quantify mAb concentrations in bio-fluid is

doi:10.6342/NTU201804105

important.

1.4 Research aim and organization of this thesis

An effective, accurate and economic bio-medical analysis method could help the physicians to get a better understanding of patients' physiological status, which may be benefit for both disease detection and drug treatment. Mass spectrometry (MS) has several advantages to perform bio-medical analysis, including the high selectivity and sensitivity. Mass spectrometry has been coupled to different kinds of separation instruments such as gas-chromatography mass spectrometry (GC-MS) and liquid-chromatography mass spectrometry (LC-MS). These techniques are frequently used to investigate the potential biomarkers for disease screening and therapeutic response. Moreover, they are also commonly adopted to measure drug concentrations in different kinds of sample matrices.[48-54] In this thesis we aimed to develop bio-analytical methods for BC detection and mAb drugs quantification by using both GC-MS and LC-MS. This thesis composed of 4 chapters, the second chapter is to find potential biomarkers for BC detection, and the third chapter is to establish the quantification approach for mAb drugs in human plasma for improving cancer treatment.

Biomarker discovery could be one of the most promising approaches to achieve early detection of BC. In the second chapter of this thesis, we aimed to find the potential

doi:10.6342/NTU201804105

biomarkers for breast cancer detection by using a GC-MS platform to analyze fatty acid profiles in both BC patients and healthy control's plasma samples, as a result, to provide an opportunity to achieve the goal of BC early detection. GC-MS has been widely applied in numerous clinical fields such as quantification of abused drugs, metabolites, and environmental pollutants. The main advantage is its' high selectivity and comprehensive spectral databases. GC-MS has been commonly used in both profiling and targeted metabolomics in recent years.[55-58] Due to the high demand for reliable quantification analysis of metabolites in complex biological matrices, in the second part of this thesis, we developed a quantitative method to investigate the potential fatty acids as the BC detection biomarkers.[59]

In the third chapter of this thesis, we developed analytical methods for therapeutic mAb quantification in human plasma samples. The first portion of the third chapter, we developed an analytical method using the protein G purification coupled to LC-MS/MS to quantify bevacizumab concentration in human plasma samples. Another IgG based mAb drug, tocilizumab, was applied as the internal standard to correct the potential variation during sample preparation. This quantification approach for quantifying bevacizumab was accurate and precise. And we also verified the applicability of this platform by quantifying the bevacizumab concentrations in patients' plasma samples. This platform could be applied to investigate the PK and PD properties of bevacizumab,

doi:10.6342/NTU201804105

and also provide the potential to perform TDM of bevacizumab drug therapy.

After the first approval of the commercial therapeutic monoclonal antibody Orthoclone OKT3 for prevention of kidney transplant rejection in 1986, the development of therapeutic mAb was growing very fast. Till the end of 2014, there were 47 therapeutic monoclonal antibody products that have been approved in the US or Europe for various diseases, and several mAbs have also been approved for other global markets.[60] As the mAb drugs have generated much interest in recent years, and the majority of these mAb drugs belong to immunoglobulin G (IgG) class, it is important to develop a general method to quantify these IgG-based therapeutic mAbs in plasma to facilitate the investigation of the their PK/PD properties.

As a result, in the second portion of the third chapter, we developed a general method to quantify the IgG base therapeutic mAb drugs, using bevacizumab, nivolumab, and pembrolizumab as the demonstration drugs. In order to make this quantification approach as a general method for IgG based drugs quantification, the sample preparation steps and internal standard selection should be universal. Protein G purification has high affinity to all the IgG class, regardless of their IgG subtypes, which is an appropriate method to trap these IgG based mAb drugs. Due to each mAb drug may have different retention times, it is impractical to purchase its' own SIL-ISs to correct the matrix effect (ME) for each analyte. As a result, we developed a general

doi:10.6342/NTU201804105

quantification method for IgG based therapeutic mAb drugs using the protein G purification coupled with a post-column infused internal standard (PCI-IS) strategy to quantify three IgG based therapeutic mAb drugs. Using a post-column infused internal standard to provide the information of matrix effects was developed several years ago, and it has been used to calibrate the ME in different types of sample matrices including urine, plasma and cerebrospinal fluid during LC-MS/MS analysis.[61-65] Because the PCI-IS can continuously reflect the different MEs encountered at each retention time point for the whole chromatographic run, one PCI-IS could be used to calibrate multiple analytes, which provides the advantages of reducing the cost of purchasing many internal standards for analyzing multiple analytes in LC-MS/MS. Considering the universal capture ability of protein G for IgG class mAbs and general calibration capability of the PCI-IS method, we anticipated that this general method will be benefit to facilitate the precision medicine for IgG based mAb therapy in the future.

To summarize, in the second chapter of this thesis, we have developed a GC-MS quantitative method to investigate the potential fatty acids as the BC detection biomarkers. In the third chapter, we developed LC-MS based analytical approaches for measuring mAb drugs concentration in human plasma. These bio-medical analytical methods could provide their potential values for clinical use, and facilitate the development of precision medicine. The conclusion and perspective of this thesis were

doi:10.6342/NTU201804105

discussed in chapter 4.

doi:10.6342/NTU201804105

Chapter 2. Using metabolomic approach for breast cancer detection

Ch2.1 Using targeted metabolomic approach for fatty acid analysis for breast cancer detection

doi:10.6342/NTU201804105

2.1.1 Introduction

Breast cancer (BC) is the most common type of cancer among women. According to WHO, there are about 570,000 women died from breast cancer, which is around 15% of all cancer deaths in women in 2015. In order to increase the survival rate, it is important to develop early detection methods, and to treat the patients at the early disease stage, which may have higher potential to cure the disease. Using biomarkers for cancer detection is a promising strategy. Previous studies have indicated that one main feature that distinguishes malignant cells from normal cells is the altered lipid metabolism, moreover, breast cancer patients present altered fatty acid profiles. Therefore, measurement of fatty acid profile shows the potential for breast cancer detection. [66-67]

Fatty acids (FAs) are aliphatic monocarboxylic acids that act as the building blocks of lipids. They play important roles in the energy balance and signal transduction in biological systems. Some fatty acids are important constituents of cell membranes [68], while others are precursors of potent bioactive lipids. [69] Imbalances in fatty acids can lead to a wide variety of diseases, including inflammation [70], neurodegeneration [71], tumorigenesis [72-73], metabolic disorders [74-75] and cardiovascular disease. [76]

Metabolomics, the study of metabolites relative to physiological state and

doi:10.6342/NTU201804105

environmental stimuli, has become increasingly important in investigations of disease pathogenesis and clinical biomarker discovery. [77] Metabolites are considered the downstream products of cellular regulatory reactions, which are necessary for normal function, maintenance or growth of a cell. [78-79] Approaches to the study of metabolomics include target analysis and global profiling. Targeted metabolomic analysis of fatty acids has indicated the potential of using fatty acids as breast cancer markers, with several free fatty acids proposed as possibly markers. [67, 80] However, one potential problem in the use of free fatty acids as cancer markers is that their concentrations may be easily influenced by dietary fat. Measurements of the total fatty acids can minimize the concentration fluctuation due to dietary fat. Therefore, the study of the total fatty acid content in breast cancer patients is required for clinical application.

Fatty acids are not naturally volatile chemicals and require chemical derivatization to improve their volatility. Although silylation reagents have been used for the derivatization of FAs, most studies employ alkylation reagent to convert polar carbonyl groups into more volatile non-polar derivatives. A wide range of alkylation reagents are available for the conversion of FAs to their corresponding fatty acid methyl esters (FAMEs). Commonly used alkylation reagents can be categorized as acid or base catalyzed methods. Acid-catalyzed methods can be used for methylating total FAs (esterified and non-esterified fatty acids), while base-catalyzed methods are not capable

doi:10.6342/NTU201804105

of derivatizing free fatty acids. [81] In addition, base-catalyzed methods consist of mild reactions suitable for methylating fatty acids bound within acylglycerides [82] in reactions requiring anhydrous conditions. On the other hand, acid-catalyzed methods can be used either in direct transesterification or in a two-step methylation including initial hydrolysis or saponification of the lipids to free fatty acids. Among the acid –catalyzed derivatization methods, acetyl chloride is the most frequently used reagent, which could be applied to direct transesterification of fatty acid. This study selected acetyl chloride derivatization method as our derivatization strategy.

This study aimed to develop an accurate, efficient, and economical GC-MS method for the plasma metabolomic study of fatty acids and apply this platform to investigate the potential biomarkers of breast cancer. We selected acetyl chloride derivatization method as our derivatization strategy and used an ionic liquid (IL) column to improve the separation efficiencies for both positional and geometrical isomers of fatty acids. The method was subjected to method-validation in terms of precision, accuracy and linearity. The validated method was applied to the investigation of potential fatty acid markers of breast cancer.

2.1.2 Experimental

doi:10.6342/NTU201804105

2.1.2.1 Chemicals and reagents

A series of fatty acid standards, including lauric acid, myristic acid, palmitic acid, elaidic acid, linoelaidic acid, arachidic acid, arachidonic acid, cis-5,8,11,14,17-eicosapentaenoic acid, behenic acid, docosapentaenoic acid, cis-4,7,10,13,16,19-docosahexaenoic acid, tricosanoic acid methyl ester, lignoceric acid, methyl nonadecanoate, methyl tricosanoate and hexacosanoic acid were obtained from Sigma-Aldrich (St. Louis, MO, US). Other fatty acid standards, including myristoleic acid, palmitoleic acid, oleic acid, α-linolenic acid, cis-11-eicosenoic acid, erucic acid, cis-13,16-docosadienoic acid, and nervonic acid were obtained from Cayman Chemical (Ann Arbor, Ml, US). Stearic acid, nonadecanoic acid, and cis-8,11,14-eicosatrienoic acid were obtained from Fluka (Buchs, St. Gallen, Switzerland). A standard mixture of 37 FAME was purchased from Supelco (Bellefonte, PA, US).

Methanol and hexane were of HPLC grade and purchased from Mallinckrodt (Phillipsburg, NJ, US). Petroleum ether was of HPLC grade and obtained from J.T. Baker (Phillipsburg, NJ, US). Potassium carbonate powder and acetyl chloride methanolic solution were purchased from ACROS (Fair Lawn, NJ, US). Sodium chloride powder was obtained from Merck (Darmstadt, Hesse, Germany).

doi:10.6342/NTU201804105

2.1.2.2 Human blood collection and sample preparation

For the metabolomic study of breast cancer, plasma samples were obtained from the National Taiwan University Hospital. The study was approved by the institutional review board of the National Taiwan University Hospital (200809033R). All plasma samples were collected after overnight fasting. Blood samples were drawn in the morning under fasting conditions and collected in citrate-containing tubes. The blood samples were centrifuged at 10,000 x g for 15 min, and the resultant plasma samples were stored at -80 ◦C until use.

For fatty acid extraction, the Folch method was applied with little modification. Fifty microliters of plasma was added to the homopolymer polypropylene vial, followed by the addition of a 2: 1 chloroform-methanol (v/v) mixture and vortexing for 10 seconds. The solution was placed on ice for 20 minutes. Subsequently, 200 μL of a 0.9% NaCl solution was added and the mixture was centrifuged at 3000 × g for 5 min. The lower layer was transferred to a glass tube, and 0.5 mL of a chloroform/methanol/deionized water (86:14:1) solution was added to the residue, repeating the extraction process. Finally, the combined lipid extracts were pooled and evaporated under an N_2 stream. The plasma extracts were stored at -20 °C until use.

Two milliliter of a methanol-hexane (4:1; v/v) mixture (containing nonadecanoic

doi:10.6342/NTU201804105

acid methyl ester (C19:0)) as the extraction internal standard) and 200 μL of 99% acetyl chloride was added to the samples, and the tubes were capped under an N_2 stream. The samples were incubated at 100°C for 30 min, and then cooled on ice. Five milliliters of a 6% K_2CO_3 solution were added to the tubes for neutralization, followed by centrifugation at 1500 × g for 5 min. The organic layers were collected from the tubes. The remaining aqueous layers were extracted with 0.5 mL of hexane. The extraction was repeated twice and the extracts were pooled and evaporated under a N_2 stream. The residue was re-dissolved in 100 μL of hexane containing 20 μg mL^{-1} of tricosanoic acid methyl ester (C23:0) as an internal standard to calibrate the GC-MS analytical error.

For direct transesterification using acetyl chloride derivatization method, 2 mL of a methanol-hexane (4:1; v/v) mixture (containing nonadecanoic acid methyl ester (C19:0)) was added in the 50 μL plasma followed by the previous mentioned derivatization procedures.

2.1.2.3. Gas chromatography–mass spectrometry analysis

All analyses were performed in split mode (1:20) on an Agilent 7890a gas chromatograph connected to an Agilent 5975C Series MSD (Agilent Technologies, Wilmington, DE, USA). The chromatographic columns were 30 m DB-5 MS+DG

doi:10.6342/NTU201804105

capillary columns (5% phenyl, 95% dimethylpolysiloxane) with an internal diameter of 250 μm (Agilent Technologies, Santa Clara, CA) and a 25 m × 0.25 mm SLB-IL82 column with a film thickness of 0.2 μm (Supelco, Bellefonte, USA). The injection volume was 1 μL. The oven temperature began at 50 °C for 1 min and was then increased to 150 °C at a rate of 30 °C/min, followed by further increases at 7 °C/min to 160 °C, 3 °C/min to 195 °C (maintained for 1 min), 6 °C/min to 200 °C and 2 °C/min to 210 °C (maintained for 1 min). The helium carrier gas flow rate was set at 1 mL/min. The GC inlets were held at 250 ℃. The equilibration time between injections was 0.5 min. The electron impact ionization was 70 eV. The MS source and MS quadrupole were maintained at 230 ℃ and 150 ℃, respectively. The masses of the analytes were acquired in full scan mode with mass range of 30-650 m/z.

2.1.2.4. Acetyl chloride derivatization method

The acetyl chloride derivatization method was employed as previously described [83]. Two mL of a methanol-hexane (4:1; v/v) mixture was added to borosilicate glass tubes containing the dried plasma extract, and the tubes were vortexed on ice.

Two-hundred microliters of 99% acetyl chloride was added to the samples, and the tubes were capped under an N_2 stream. The samples were incubated at 100°C for 30

doi:10.6342/NTU201804105

min, and then cooled on ice. Five milliliters of a 6% K_2CO_3 solution were added to the tubes for neutralization, followed by centrifugation at 1500 × g for 5 min. The organic layers were collected from the tubes. The remaining aqueous layers were extracted with 0.5 mL of hexane. The extraction was repeated twice and the extracts were pooled and evaporated under a N_2 stream. The residue was re-dissolved in 100 μL of hexane containing 20 μg mL^{-1} of tricosanoic acid methyl ester (C23:0) as an internal standard.

2.1.2.5 Data processing

Peak areas were calculated by integrating the respective extracted ion chromatograms (EIC) with Agilent ChemStation software (Santa Clara, CA, US). Numerical data were exported and processed with Microsoft Excel 2007. The paired *t* test was employed to identify significant fatty acids between control and breast cancer patients. The generation of principal component analysis (PCA) plots and barplots were carried out in *R* (Version 2.12.2). The receiver operating characteristic (ROC) curve analysis was performed using Stata/SE11-2 for Windows (StataCorp LP, College Station, TX, USA).

2.1.3 Results and discussion

doi:10.6342/NTU201804105

2.1.3.1 Development of the GC-MS method

Fatty acids are composed of organic chains of various lengths. These chains also vary in the number and locations of double bonds. As some positional and geometrical isomers show similar fragmentation patterns by mass spectrometry, good chromatographic resolution is required to provide additional retention time information for use in compound identification. A thirty-seven-component fatty acid methyl ester (FAME) mixture was used for selectivity optimization.

Recently, ionic liquid (IL) columns have been introduced for the separation of FAME mixtures and were found to give better selectivity for FAME mixtures compared with wax or cyanopropylsiloxane columns [84-85]. SLB-IL-82 is an ionic liquid column containing asymmetrical organic cations and inorganic anions. Unlike a traditional polysiloxane phase, the ionic liquid column provides good separation for both polar and nonpolar components. Additionally, it is able to distinguish different positional and geometrical isomers [86]. Several studies have indicated that the column bleed of SLB-IL 82 is lower than found for other polar columns [87]. This advantage is particularly important for the metabolic profiling of fatty acids in biological specimens because some fatty acids appear in only trace amounts. This low column bleed results in relatively low background noise, which improves the identification and quantification

doi:10.6342/NTU201804105

accuracy for low-abundance fatty acids.

Considering the potential differences in the biological functions of the fatty acids isomers, we adjusted the temperature gradient to separate positional and geometric fatty acid isomers. A detailed description of the optimized gradient program was described in Section 2.1.2.3. The total runtime of the optimized GC method was 26 minutes. While most fatty acids in human plasma have cis configurations, C18:1n9t and 18:2n6t are the most commonly detected trans fatty acids in human plasma. Figure 2.1 a revealed that the SLC-IL82 column, in conjunction with the optimized conditions, could separate trans fatty acids from their respective cis isomers. The optimized conditions also provided good selectivities for fatty acids in the plasma samples (Figure 2.1 b).

2.1.3.2 Method validation

Before validating the acetyl chloride derivatization method in conjunction with GC-MS analysis for fatty acid profiling, we additionally determined the shortest reaction time required for acetyl chloride derivatization. Almost all of the previous studies used 1 hour reaction times for the transesterification of fatty acids [83, 88]. After evaluation of several transesterification reaction times, we found that reactions run for

doi:10.6342/NTU201804105

30 minutes provided comparable signal intensities to those run for 1 hour for both fatty acid standard solutions and plasma samples when using a direct transesterification method (without lipid extraction). Therefore, a 30 minute reaction time with acetyl chloride (using direct transesterification) followed by GC-MS analysis was validated in terms of precision, accuracy and linearity.

Twenty-four fatty acid standards were used for method validation. Tricosanoic acid methyl ester was used as the internal standard. The intra-day (n = 9) and inter-day (n = 3) precision for each of the fatty acids in terms of peak area ratio were tested three times a day for 3 days at low, medium and high levels of their respective linear ranges (Table 2.1.1). With the exception of lauric acid (C12:0), both the intra-day and inter-day precisions of all of the tested fatty acids had relative standard deviations (RSDs) of less than 10% (Table 2.1.1).

The linear ranges for each fatty acids were designed according to their respective biological concentrations. Table 2.1.2 shows the calibration curves and the correlation coefficients of each fatty acid. The test results revealed correlation coefficients higher than 0.999 for all of the fatty acids except C12:0 and C20:0 within the tested ranges. Quantification accuracy was evaluated at three concentrations (Table 2.1.2). Over 90% of the tested results showed recoveries within 85%-115%.

doi:10.6342/NTU201804105

2.1.3.3 Fatty acid profiling in breast cancer research

Breast cancer is one of the most common cancers observed among women, and its associated death rate has been on the rise. In 2013, there were approximately 232,340 new cases of invasive breast cancer, and 39,620 breast cancer deaths are expected among US women. It was estimated that one in 8 women in the United States will develop breast cancer in her lifetime [89]. Due to the high incidence rate of breast cancer, the development of a high-quality screening method is urgently needed.

Targeted metabolomic analysis of fatty acids has indicated the potential of using fatty acids as breast cancer markers [67, 80]. Compared with previous studies on breast cancer, this study measured the total fatty acids (free and esterified fatty acids) with more comprehensive investigation of fatty acid species. Considering the potential differences in biological functions of fatty acids isomers, positional and geometric fatty acid isomers were studied separately.

We collected plasma samples from 50 healthy volunteers and 48 breast cancer patients to investigate potential fatty acid markers. The clinical characteristics of these healthy volunteers and breast cancer patients were listed in Table 2.1.3. To minimize biased comparison, samples obtained from pre-menopausal and post-menopausal women were separately compared. All of the plasma samples were derivatized by the

doi:10.6342/NTU201804105

acetyl chloride method followed by GC-MS analysis.

The fatty acid profiling results revealed that the concentrations of three fatty acids including C22:0, C24:0, C18:2n6 were significantly lower in post-menopausal breast cancer patients ($P<0.05$). In addition to C22:0, C24:0, C18:2n6, seven fatty acids, including C16:0, C18:0, C18:1n9c, C20:0, C20:4n6, C22:6n3, and C24:1n9, showed significant differences in pre-menopausal patients. Decreased serum levels of free fatty acids have been found to be associated breast cancer in most studies. This study found the trend of total fatty acid expression in breast cancer patients to be very similar to free fatty acids in that all fatty acids were expressed in lower quantities in breast cancer patients. The decrease of fatty acids has been proposed to result from the fact that fatty acids are rapidly metabolized in cancer patients for the synthesis of membrane phospholipids[66].

Compared with the post-menopausal group, greater quantities of significant fatty acids were observed in the pre-menopausal group. The incidence rates of breast cancer in Taiwan tend to be more prominent among a younger age group. The number of patients in Taiwan under the age of 40 accounts for 16.6% of the total breast cancer population, whereas the same demographic in the United States accounts for only 8%. Moreover, variations in in the dominant molecular subtype (luminal or basal-like) are found between young Taiwanese women and young breast cancer patients in the west[90].

doi:10.6342/NTU201804105

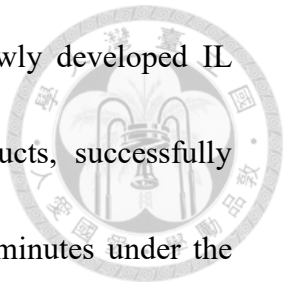
This observation suggests a more complicated pathophysiological origin for young breast cancer patients in Taiwan. Our study observed complicated changes in the fatty acid profiles of young breast cancer patients, which may provide useful insight into the pathophysiological origin of breast cancer.

A binary logistic regression model was built using the significant fatty acids to test their power in predicting breast cancer. The ROC curve was computed for the logistic regression model (Figure 2.1.2), and the model was generated through different combinations of significant fatty acids to give the highest AUC value using the minimum number of fatty acid species. When combining fatty acids C22:0 and C24:1n9 for pre-menopausal group and combining fatty acids C18:2n6, C22:0 and C24:0 for post-menopausal group for the prediction of breast cancer, the AUC values were 0.78 (73.3% sensitivity and 70.8% specificity) and 0.72 (70.6% sensitivity and 70.4% specificity). The distribution of the fatty acids used to build the prediction model are provided in Table 2.1.4.

2.1.4 Conclusions

An accurate, efficient and economic method for the employment of plasma metabolomics of fatty acids was proposed in this study. Acetyl chloride method was

doi:10.6342/NTU201804105



demonstrated to provide the good derivatization efficiency. A newly developed IL column, SLB-IL 82, was used to analyze the derivatized products, successfully separating positional and geometric fatty acid isomers within 26 minutes under the optimized conditions. The proposed method was applied in the investigation of potential breast cancer markers. Three (C18:2n6, C22:0, C24:0) and ten fatty acids (C16:0, C18:0, C18:1n9c, C18:2n6, C20:0, C20:4n6, C22:0, C22:6n3, C24:0, C24:1n9) were found to be significantly decreased in post- and pre-menopausal breast cancer patients, respectively. Nevertheless, further investigations using larger populations are needed to adequately prove these potential markers to be viable for clinical application. These fatty acids represented as potential biomarkers for early detection of BC and to reduce BC mortality.

In addition to early detection, providing appropriate drug treatment could also reduce cancer mortality. Measurement of drug concentrations in bio-fluid would improve efficacy and safety. Due to the growing importance of mAb in cancer therapy, in the next chapter, we developed two LC-MS/MS based analytical methods to quantify the therapeutic mAb drugs in human plasma samples.

doi:10.6342/NTU201804105

Table 2.1.1 Precision of 24 fatty acids at three concentrations.

Fatty acid	Repeatability (RSD%)			Intermediate precision (RSD%)		
	Low	Medium	High	Low	Medium	High
C12:0	13.24	1.55	4.47	20.91	5.30	6.60
C14:0	2.39	1.28	0.89	3.08	2.12	8.06
C14:1	4.03	1.24	2.15	4.28	2.78	8.46
C16:0	1.30	2.02	4.09	2.55	4.33	7.72
C16:1	1.10	0.97	1.53	5.34	2.42	3.26
C18:0	1.52	0.52	0.58	3.24	6.35	3.77
C18:1n9c	0.56	0.43	1.57	7.94	8.79	1.75
C18:1n9t	0.77	1.08	0.66	2.73	2.55	2.11
C18:2	1.28	2.07	0.81	2.67	8.06	4.55
C18:3n	4.26	1.25	3.81	4.17	1.12	6.45
C20:0	1.75	2.09	0.70	3.33	3.53	1.96
C20:1	1.43	1.15	3.04	2.98	2.71	4.69
C20:2	3.37	0.67	0.39	6.80	3.31	5.36
C20:3n6	1.42	0.36	0.85	1.38	9.40	1.19
C20:4	1.62	0.68	1.10	1.61	1.53	4.80
C20:5	2.95	0.49	1.00	5.73	5.87	6.60
C22:0	0.78	1.55	0.26	2.36	6.41	1.63
C22:1	0.42	1.01	2.98	9.08	1.39	3.71
C22:2	4.89	2.17	1.18	5.17	2.29	1.88
C22:5	7.21	2.01	1.02	7.75	7.30	6.63
C22:6	1.19	0.80	1.15	3.50	9.06	5.37
C24:0	1.79	0.72	0.53	8.88	5.90	7.26
C24:1	7.70	0.73	0.43	8.83	3.66	6.34
C26:0	1.76	0.56	1.85	2.43	6.16	7.48

doi:10.6342/NTU201804105

Table 2.1.2 Linearity and accuracy of 24 fatty acids at three concentrations.

FAME	Calibration range (μg/mL)	Linearity	Accuracy (%)		
			High	Medium	Low
C12:0	1.5-50	0.998	100.0 ± 9.2	104.9 ± 11.4	93.0 ± 20.6
C14:0	5-200	0.999	95.8 ± 3.1	104.4 ± 2.3	103.0 ± 3.4
C14:1	10-200	0.999	111.6 ± 15.8	101.9 ± 6.0	86.8 ± 8.1
C16:0	31.3-500	0.999	92.1 ± 3.2	103.8 ± 1.5	87.9 ± 1.9
C16:1	5-200	0.999	96.7 ± 8.3	90.3 ± 1.8	104.4 ± 0.8
C18:0	31.3-500	0.999	100.3 ± 0.7	105.1 ± 7.2	105.8 ± 5.0
C18:1n9c	31.3-500	0.999	93.7 ± 2.3	102.2 ± 1.6	90.6 ± 1.3
C18:1n9t	31.3-1000	0.999	119.3 ± 18.3	120.9 ± 3.6	101.0 ± 0.5
C18:2	15.6-500	0.999	93.5 ± 0.5	103.2 ±1.2	81.0 ± 0.2
C18:3n	1.5-50	0.999	83.7 ± 10.1	101.2 ± 0.4	109.1 ± 19.5
C20:0	1.5-50	0.998	85.6 ± 11.2	110.5 ± 6.5	117.6 ± 10.8
C20:1	1.5-100	0.999	114.2 ± 23.4	120.0 ± 18.9	81.9 ± 9.5
C20:2	1.5-50	0.999	99.9 ± 5.5	106.1 ± 2.2	114.6 ± 3.1
C20:3n6	15.6-500	0.999	104.1 ± 12.6	103.1 ± 0.8	86.1 ± 6.2
C20:4	31.3-500	0.999	89.5 ± 7.9	85.9 ± 10.7	94.5 ± 10.3
C20:5	5-200	0.999	108.7 ± 16.1	113.7 ± 4.4	108.5 ± 17.4
C22:0	1.5-100	0.999	115.2 ± 3.6	102.5 ± 1.5	97.9 ± 1.4
C22:1	15.6-500	0.999	120.9 ± 13.8	91.7 ± 9.0	75.9 ± 16.1
C22:2	1.5-50	0.999	98.5 ± 6.7	87.4 ± 8.1	90.8 ± 14.7
C22:5	5-200	0.999	90.9 ± 1.2	99.8 ± 11.3	109.8 ± 1.1
C22:6	31.3-500	0.999	100.1 ± 8.1	92.1 ± 8.5	100.8 ± 4.7
C24:0	1.5-50	0.999	113.3 ± 26.6	109.7 ± 11.9	127.0 ± 13.8
C24:1	1.5-50	0.999	104.0 ± 3.5	104.6 ± 1.5	118.3 ± 12.4
C26:0	31.3-500	0.999	97.8 ±7.8	89.9 ± 1.4	85.5 ± 1.2

doi:10.6342/NTU201804105

Table 2.1.3 Clinical characteristics of study subjects

	Healthy control		Breast cancer patient	
	Pre-menopausal	Post-menopausal	Pre-Menopausal*	Post-menopausal
Number	23	27	31	17
Age	43.1 ± 5.2	59.4 ± 7.0	43.4 ± 5.4	58.4 ± 7.3
Height (cm)	157.7 ± 4.8	154.8 ± 3.3	158.2 ± 5.4	154.8 ± 4.7
Weight (kg)	56.1 ± 7.6	56.9 ± 6.9	59.1 ± 12.2	59.5 ± 11.1
BMI (kg/m^2)	22.6 ± 2.7	23.7 ± 2.8	23.6 ± 4.6	24.9 ± 4.8
Stage I			11	9
Stage II			15	6
Stage III			4	2

*Stage information was missing in one pre-menopausal patient

doi:10.6342/NTU201804105

Table 2.1.4 The distribution of fatty acids used to build the binary logistic regression model

Breast cancer patient			
Pre-menopausal (μg mL^{-1})		Post-menopausal (μg mL^{-1})	
C22:0	12.5 ± 2.6	C18:2n6	979.6 ± 257.1
C24:1n9	20.9 ± 5.3	C22:0	13.4 ± 3.0
		C24:0	10.3 ± 2.2
Healthy control			
Pre-menopausal (μg mL^{-1})		Post-menopausal (μg mL^{-1})	
C22:0	14.2 ± 3.3	C18:2n6	1118.0 ± 306.8
C24:1n9	24.7 ± 8.2	C22:0	15.2 ± 3.8
		C24:0	11.8 ± 2.2

doi:10.6342/NTU201804105

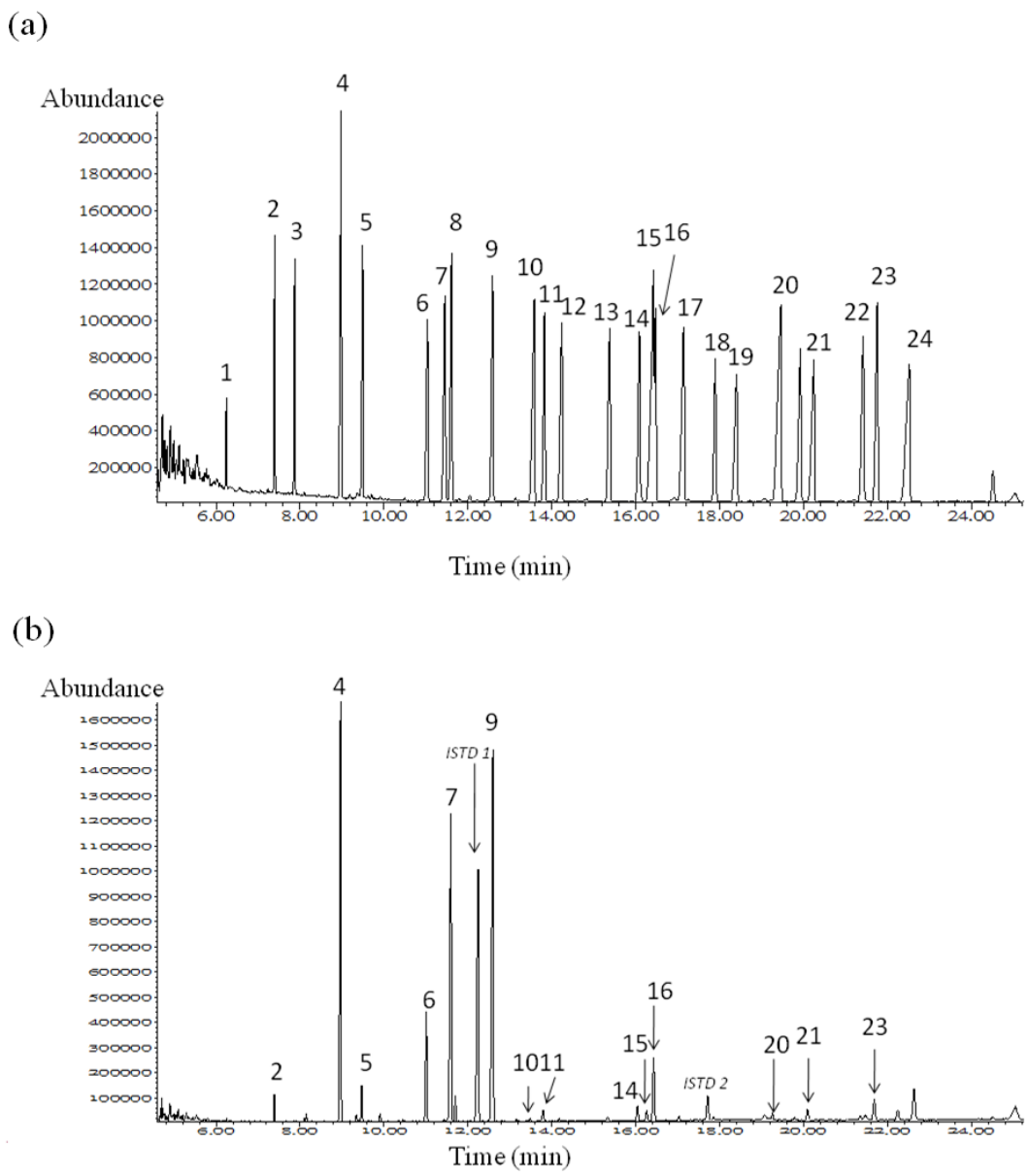


Figure 2.1.1 GC-MS chromatograms of (a) FAME standard and (b) a breast cancer patient's plasma extract analyzed by the SLC-IL 82 under the optimized GC-MS conditions. (The detailed conditions were in Section 2.2.3) (peak identification: **1**: C12:0, **2**: C14:0, **3**: C14:1, **4**: C16:0, **5**: C16:1, **6**: C18:0, **7**: C18:1n9c, **8**: C18:1n9t, **9**: C18:2, **10**: C20:0, **11**: C18:3, **12**: C20:1, **13**: C20:2, **14**: C20:3, **15**: C22:0, **16**: C20:4, **17**: C22:1, **18**: C20:5, **19**: C22:2, **20**: C24:0, **21**: C24:1, **22**: C22:5, **23**: C22:6, **24**: C26:0, *ISTD 1*: C19:0, *ISTD 2*: C23:0)

doi:10.6342/NTU201804105

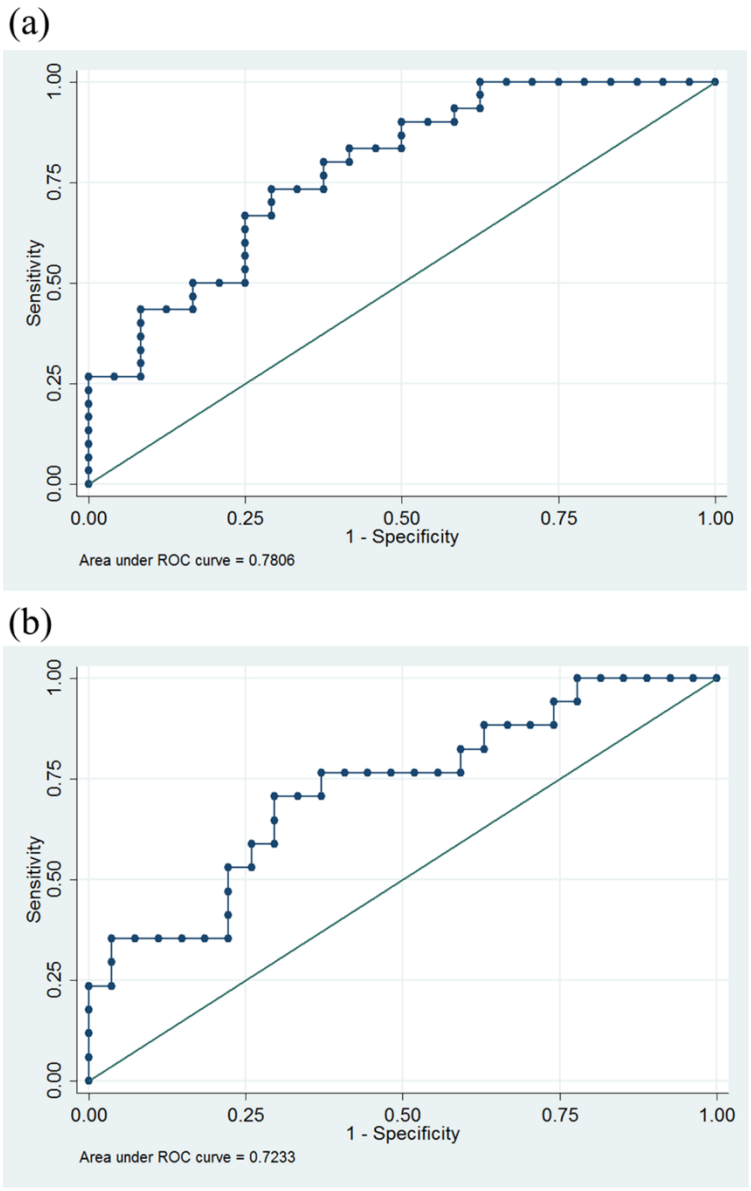


Figure 2.1.2 ROC analysis for the discrimination of breast cancer and control groups in (a) pre-menopausal (C22:0, C24:1n9) and (b) post-menopausal women (C18:2n6, C22:0, C24:0).

doi:10.6342/NTU201804105

Chapter 3.

Development of LC-MS/MS methods for IgG based therapeutic monoclonal antibodies quantification

Ch3.1 Development of an LC-MS/MS method with protein G purification strategy for quantifying bevacizumab in human plasma

Ch3.2 A general method for quantifying IgG-based therapeutic monoclonal antibodies in human plasma using protein G purification coupled with a two internal standard calibration strategy using LC-MS/MS

doi:10.6342/NTU201804105

Ch3.1 Development of an LC-MS/MS method with protein G purification strategy for quantifying bevacizumab in human plasma

doi:10.6342/NTU201804105

3.1.1 Introduction

Biopharmaceutical products such as protein drugs and monoclonal antibodies are currently of great interest. These types of drugs have the potential to improve therapeutic effects and reduce side effects. Monoclonal antibody (mAb) drugs are one of the rapidly growing categories of biopharmaceutical products. From 2010 to 2014, greater than 30% of the approved biopharmaceuticals in the United States and Europe belong to mAb-based products.[91] Bevacizumab is a humanized immunoglobulin G (IgG) monoclonal antibody (mAb) drug that against human vascular endothelial cell growth factor A (VEGF-A), and it is also the first anti-angiogenesis agent to be approved by the FDA for metastatic colorectal cancer treatment in 2004.[92] Subsequently, bevacizumab has been approved for treating many types of cancers, such as non-small cell lung cancer, metastatic renal cell carcinoma, and glioblastoma.

Personalized medicine is increasingly important in the clinic, and therapeutic drug monitoring (TDM) is one important strategy to achieve personalized medicine. TDM of monoclonal antibody concentrations has also been conducted to improve therapeutic efficacy, reduce side effects and improve the control of disease activity.[93] A previous study found that pharmacokinetic (PK) behaviors are usually more complex with biopharmaceutical products than with conventional small molecule drugs.[94] Although

doi:10.6342/NTU201804105

mAbs can provide a better safety profile, a recent study indicated that clinical outcomes vary among patients treated with bevacizumab, and the extent of the side effects such as vascular disorders also varied.[95] As a result, simple and accurate quantification methods for these biopharmaceutical drugs in biological fluids is vital and allows dose adjustment depending on the pharmacokinetic profiles of different patients.

Currently, ligand binding assay (LBA), for example, enzyme linked immunosorbent assay (ELISA) is the most widely used methods for the measurement of mAb concentrations in plasma/serum.[96-98] However, these LBA methods have several limitations. First, the customized of specific reagents such as antigens or antibodies are usually required for LBA assays, and the reagent's quality and consistency between different batches would seriously affecting the method performances including specificity, robustness, and also sensitivity.[99-100] Second, the generation of the specific reagent for LBA is commonly time and cost consuming. [101] Third, the interference in the sample matrix may also lead to inaccurate quantification and insufficient dynamic range due to the potential cross reaction.[102-103] A recent study compared the accuracy between LBA and LC-MS for infliximab quantification in human plasma. A significant bias was found with commercial ELISA.[104] Therefore, to develop alternative methods for accurate quantification of mAbs is important in clinical. Due to its high sensitivity and selectivity, LC-MS is being used increasingly for

doi:10.6342/NTU201804105

quantifying monoclonal antibodies in human plasma.[105-107] However, serious matrix effects may lead to inaccurate quantification. Therefore, many sample cleanup methods have been proposed for purifying mAbs from biological matrixes.[108-111] Iwamoto et al. developed a nano-surface and molecular-orientation limited (nSMOL) proteolysis method for quantification of bevacizumab in human plasma; however, it required a special device. Also, clinical validation was not performed in their study for evaluation the utility of their method in clinical measurement.[108] Todoroki recently used an anti-idiotype antibody to purify bevacizumab from human plasma followed by LC with fluorimetric detection analysis. Since idiotype antibodies are generated by a customized service, the sample preparation cost would be relatively higher.[112] Therefore, a simple and cost-effective method is still required to enable a wider application of TDM in clinical fields.

In this study, we proposed a general purification method coupled to LC-MS/MS to determine bevacizumab concentrations in human plasma. Protein G magnetic beads were chosen for sample purification due to their wide availability and low cost. Protein G magnetic beads selectively trap IgG class antibodies in human plasma. This procedure can help to reduce the sample matrix complexity and the large amount of endogenous proteins such as albumin and other proteins, including immunoglobulin A and immunoglobulin M, can be removed. To calibrate the potential loss by trapping,

doi:10.6342/NTU201804105

another IgG-based pharmaceutical product, tocilizumab, was used as an internal standard. The tandem MS displayed the great selectivity for the surrogate peptide quantification in human plasm. This LC-MS/MS method was validated in term of its precision, accuracy, linearity and sensitivity. Finally, the validated method was used to analyze plasma samples obtained from breast cancer patients with brain metastasis to demonstrate its usefulness for TDM in clinical fields.

3.1.2 Materials and methods

3.1.2.1 Reagents and materials

Bevacizumab was purchased from Roche Applied Science (Indianapolis, IN, USA). Trypsin was purchased from Promega (Madison, WI, USA). Protein G Mag Sepharose Xtra beads were purchased from GE (Piscataway, NJ, USA). MS-grade methanol was purchased from Scharlau Chemie (Sentmenat, Barcelona, Spain). Acetonitrile (ACN) was obtained from J.T. Baker (Phillipsburg, NJ, USA). Ammonium bicarbonate, formic acid (FA) solution (99%), dithiothreitol (DTT), and iodoacetamide (IAA) were purchased from Sigma (St. Louis, MO, USA). Sodium chloride and sodium phosphate dibasic anhydrous were obtained from J.T. Baker (Phillipsburg, NJ, USA). Potassium chloride was purchased from Fluka (Buchs, St. Gallen, Switzerland). Potassium phosphate monobasic was obtained from Sigma (St. Louis, MO, USA). The

doi:10.6342/NTU201804105

extend SIL-IS was synthesized by GenScript (Piscataway, NJ, USA). Tocilizumab was purchased from Genentech (South San Francisco, CA, USA).

3.1.2.2 LC-MS/MS system

An Agilent 1290 UHPLC system equipped with an Agilent 6460 triple quadrupole system (Agilent Technologies, Waldbronn, Germany) was used for the analysis. An Aeris™ PEPTIDE XB-C18 100 x 2.1 mm (1.7 μm) column (Phenomenex, Torrance, USA) was selected for the separation. The mobile phase consisted of 0.1% aqueous formic acid (solvent A) and 0.1% formic acid in ACN (solvent B) at a flow rate of 0.3 mL min^{-1}. The gradient profile was the following: 0-1.5 min, 5% B; 1.5-5 min, 5-50% B; 5-5.5 min, 50-100% B; 5.5-6.5 min, 100% B; and column re-equilibration with 5% B for 2 min. The sample reservoir was maintained at 4 °C, and the column oven was set at 40 °C. The injection volume was 20 μL. A positive electrospray ionization mode was utilized with the following parameters: a 325 °C dry gas temperature, a 7 L min^{-1} dry gas flow rate, a 45 psi nebulizer pressure, a 325 °C sheath gas temperature, an 11 L min^{-1} sheath gas flow rate, a 3500 V capillary voltage, and a 500 V nozzle voltage. MS acquisition was executed in multiple reaction monitoring (MRM) mode. The transitions for surrogate peptides were m/z 588.3→602.3 and 523.3→797.4, and the transition for extended stable isotope-labeled

doi:10.6342/NTU201804105

peptide was 590.3→602.3. The transition for tocilizumab was 514.8→526.25.

3.1.2.3 Trap and digestion of bevacizumab from human plasma

In this study, we used protein G beads to –trap bevacizumab from human plasma. The protein G bead solution was first conditioned with 200 μL of phosphate buffered saline (PBS) buffer twice. Next, 5 μL of plasma and 20 μL of tocilizumab (50 μg mL^{-1}) were added to the bead solution and incubated for 1 hour at 4 °C. After the incubation, 200 μL of PBS buffer and 200 μL of deionized water were used as washing solutions to remove unbound proteins. To elute the bevacizumab from the beads, 200 μL of 100 mM formic acid solution was added to the sample twice. The eluent was dried under N_2. The dried eluent was reconstituted with 150 μL of 100 mM ammonium bicarbonate buffer and 150 μL of deionized water, and the solution was heated at 90 ˚C for 25 min. Ten microliters of 100 mM DTT was added to the solution and then heated at 60˚C for 60 min. After cooling, 10 μL of 100 mM IAA was added for alkylation at 30˚C for 30 min in the dark. For trypsin digestion, 18 μL of trypsin (20 μg/ 200 μL) and 20 μL of 100 mM ammonium bicarbonate were added to the solution and heated at 37˚C for 12 hours. To terminate the digestion, 20 μL of 10% FA was added to the trypsin digestion solution. The digested sample was subjected to LC-MS/MS analysis. To evaluate the calibration performance, ten microliters of extended stable isotope-labeled internal

doi:10.6342/NTU201804105

standard (SIL-IS) (10 μg/mL) was added before trypsin added.

3.1.2.4 Method validation

3.1.2.4.1 Selectivity

Six plasma blank samples (without administration of bevacizumab and tocilizumab) were applied to evaluate the selectivity of this method. We compared the chromatograms of plasma blank and bevacizumab and tocilizumab spiked plasma to confirm whether there were any interference exist at the same retention time.

3.1.2.4.2 Linearity, limits of detection (LOD), limits of quantification (LOQ)

A series dilution of the stock solution (25000 μg mL^{-1}) was conducted to produce solution concentrations ranging from 1000 μg mL^{-1} to 30 μg mL^{-1} of bevacizumab in human plasma samples. Aliquots of bevacizumab solution were added to plasma blank to obtain 30, 50, 100, 300, 500, 1000 μg mL^{-1} spiked samples to generate the calibration curve, and each concentration was analyzed for three replicates. The peak area of the analyte was integrated using Agilent software. The calibration curve was obtained with a weighting factor of 1/X and by linear regression analysis. The limit of detection (LOD) was defined as a signal to noise (S/N) ratio of three. The limit of

doi:10.6342/NTU201804105

quantification (LOQ) was defined as a signal to noise (S/N) ratio of ten.

3.1.2.4.3 Accuracy and precision

To evaluate accuracy, three different concentrations (30, 300 and 1000 μg mL^{-1} in plasma) of bevacizumab were spiked into the different plasma samples from healthy controls and patients with infection, and the back calculated concentrations were compared to the spiked concentrations. Five determinations per concentration were applied to evaluate the accuracies according the FDA guidance for bioanalytical method validation. For repeatability evaluations, the samples were analyzed by five determinations per concentration within the same day. For intermediate precision, three samples per concentration were prepared individually and analyzed at three different days.

3.1.2.4.4 Stability, matrix effect and extraction recovery

Processed sample stability was evaluated by measuring the sample concentration after 24 hours kept in 4 °C autosampler. To measure the matrix effect, three concentrations of digested bevacizumab standards were individually spiked into six plasma blank samples and one reagent blank sample. The matrix effects at three concentrations were calculated using surrogate peptide intensity in plasma blank

doi:10.6342/NTU201804105

divided by surrogate peptide intensity in reagent blank. The reagent blank was composed of same component as the bevacizumab digestion buffer.

To evaluate the extraction recovery, bevacizumab was spiked into the plasma samples at three different concentrations (30, 300 and 1000 μg mL^{-1} in plasma) before incubation with protein G beads as the pre-spiked samples. The post-spiked samples were prepared by adding bevacizumab into the plasma blank samples after the protein G trapping step. The extraction recoveries were calculated by dividing the peak area for the pre-spiked sample by the peak area for the post-spiked sample and multiplying by 100%.

3.1.2.5 Collection of clinical samples

Plasma samples were collected at the National Taiwan University Hospital. The study was approved by the institutional review board of the National Taiwan University Hospital (201709061RIND). Avastin® (bevacizumab) is administered intravenously at a dose of 15 mg kg^{-1}. The blood samples were centrifuged at 10,000 × g for 15 min, and the resultant plasma samples were stored at −80 °C until use.

doi:10.6342/NTU201804105

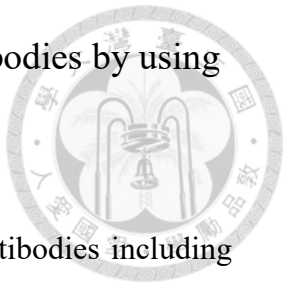
3.1.3 Results and discussion

3.1.3.1 Method development

3.1.3.1.1 Selection of surrogate peptides of bevacizumab

This study used surrogate peptides for bevacizumab quantification. Plasma blank and bevacizumab-spiked samples were profiled by time of flight mass spectrometry to identify unique peptides that were contributed by bevacizumab. The amino acid sequences of surrogate peptides were investigated using the website PeptideMass (http://web.expasy.org/peptide_mass/). Two surrogate peptides, VLIYFTSSLHSGVPSR and FTFSLDTSK, were identified, and they were further investigated for their mass transition for bevacizumab quantification. The surrogate peptide (VLIYFTSSLHSGVPSR) with higher abundance was used for quantification, and the other surrogate peptide (FTFSLDTSK) was used for confirmation. The amino acid sequences, mass transitions and the corresponding mass parameters are listed in Table 3.1.1 These surrogate peptides sequences of bevacizumab were the same as the previous studies. [108] [113] Figure 3.1.1 shows that the selected surrogate peptides were selective and that no interference could be detected.

doi:10.6342/NTU201804105



3.1.3.1.2 Trapping of immunoglobulin G monoclonal antibodies by using protein G beads

Protein G beads were used to selectively extract IgG class antibodies including bevacizumab. A schematic diagram for protein G trapping procedure is shown in Figure 3.1.2. The entire immunoglobulin G trapping process can be divided into three steps: incubation, wash and elution. Bevacizumab in human plasma was first trapped using protein G beads with an end-to-end mixer to obtain sufficient interactions between the protein G beads and bevacizumab. Phosphate buffered saline (PBS buffer) was then used to remove unbound interfering substances. Finally, an acid solution (100 mM formic acid) was added to disrupt the binding between the protein G beads and the target analyte. This immune-affinity trapping workflow provides a convenient method for reducing the complexity of the sample matrix.

3.1.3.1.3 Optimization of the protein G trapping procedure

To effectively trap bevacizumab from plasma samples, we evaluated different protein G bead volumes for extraction of bevacizumab in 5 μL of plasma sample. Theoretically, the capacity of 40 μL of protein G beads is sufficient to bind all of the endogenous IgG and bevacizumab in plasma samples (3.5 μg of human IgG per μL of 10% medium slurry, according to the manufacturer). Five different plasma bead volume

doi:10.6342/NTU201804105

ratios of 1:2, 1:4, 1:8, 1:12 and 1:16 were investigated to determine the optimal volume of protein G beads required to provide sufficient capacity for the bevacizumab in the samples. The samples were incubated at 4°C with end-to-end rotation. The results are shown in Figure 3.1.3 (A) When the ratio was lower than the theoretical capacity (1:8), the signal intensity of the surrogate peptide was relatively low with a high standard deviation. The peak area of the surrogate peptide reached a plateau when the ratio was above 1:8 and was without improvement when the ratio was increased to 1:12 and 1:16. As a result, we selected 1:8 as the optimal plasma: bead reaction ratio to trap bevacizumab from the plasma samples.

The incubation time for protein G beads and plasma samples was further evaluated. To evaluate the time effect on the trapping efficiency, we tested four incubation times from 30 to 180 minutes. As shown in Figure 3.1.3 (B), the abundance of surrogate peptide clearly increased from 30 to 60 minutes; however, as the incubation time increased, the amount of surrogate peptide did not increase noticeably. To provide an efficient method, we selected 60 minutes as the incubation time.

Other parameters, such as buffer volume and manner of incubation, were also optimized to effectively trap bevacizumab from human plasma samples. A large volume of buffer (420 μL) with end-to-end rotation was found to provide better trapping performance due to the more thorough contact between the protein G beads and

doi:10.6342/NTU201804105

bevacizumab.

3.1.3.1.4 Optimization of the trypsin digestion procedure

To acquire the optimal trypsin digestion results, we investigated the digestion time and the trypsin: protein ratio. For the evaluation of the digestion time, we tested five digestion times from 1 hour to 16 hours. We observed that from 1 hour to 12 hours, the abundances of surrogate peptide clearly increased; however, as the incubation time increased, there was no enhancement of the abundance of surrogate peptide (Figure 3.1.4 A). Therefore, 12 hours was selected as the digestion time.

The trypsin: protein ratio is also a critical parameter that should be considered in order to provide better digestion efficiency. A trypsin: protein ratio from 1:20 to 1: 100 (w/w) is frequently recommended for these digestions. The protein amount is calculated using an average value of IgG among general adult populations.[114] This study evaluated trypsin: protein ratios from 1:10 to 1:100, and the results are shown in Figure 3.1.4 B. The results indicate that the surrogate peptide abundances for trypsin: protein ratios of 1:10 and 1: 20 were significantly lower than for a trypsin: protein ratio of 1:50. However, when the ratio was increased to 1:100, the trypsin amount was insufficient to digest the target analyte in plasma samples. To provide a sensitive and robust quantification method, a 1:50 ratio of trypsin: protein was selected.

doi:10.6342/NTU201804105

3.1.3.1.5 Selection of internal standards for improving quantification accuracy

LC-MS/MS protein quantification studies commonly use extended SIL-peptides as internal standards (IS).[110, 115] However, extended SIL-peptides could not be trapped by protein G beads and a potential trapping loss therefore could not be calibrated. Hongyan *et al* suggested the use of the entire molecule as the internal standard to calibrate fluctuations in the sample processing steps.[111] Consequently, we compared two types of internal standards for their correction efficiency: extended-SIL peptide-IS and an IgG-based drug-IS. Three concentrations of quality control (QC) samples were used to evaluate the calibration performance. For IgG-based drug-IS, we adapted another IgG based drug, tocilizumab, as the internal standard to correct for a possible preparation error from the bead trapping step to trypsin digestion. The sequence and the corresponding mass parameters of two types of internal standards are listed in Table 3.1.1. Both MRM chromatograms for these internal standards in plasma blank samples and plasma spiked samples are shown in Figures 3.1.1 C and 3.1.1 D. There was no interference with these transitions for the two internal standard peptides. A comparison of the correction efficiency is shown in Figure 3.1.5. Both quantification accuracies and precision were used to evaluate the correction efficiency of different internal standards.

doi:10.6342/NTU201804105

As shown in Figure 3.1.5 A, without applying any internal standard correction, the biases were higher than 40% at low and high concentrations. When using the extended SIL-peptide as the internal standard, the accuracy was not noticeably improved. On the other hand, when using the tocilizumab as the internal standard, the bias of all concentration levels were decreased to less than 15%. We additionally compared the precision of peak areas obtained from three replicate samples. As shown in Figure 3.1.5 B, the results indicated that without applying any internal standard correction, the RSD is approximately 15-30% for different concentrations. When using the extended SIL-peptide as the internal standard, the RSD was not noticeably decreased; in the low and high concentration groups, the RSDs were even greater. On the other hand, when using tocilizumab as the internal standard, the RSD significantly decreased to less than 12%. The good correction efficiency of tocilizumab could be attributed to both tocilizumab and bevacizumab belong to the IgG class and also present in intact form, and as a result, the preparation including protein G trapping and digestion efficiency may be mimicked. According to accuracy and precision comparison results, we could conclude that tocilizumab represents as an effective internal standard for bevacizumab quantification.

doi:10.6342/NTU201804105

3.1.3.2 Method validation

3.1.3.2.1 Selectivity

Selectivity was evaluated using six plasma samples obtained from healthy controls and patients. Chromatograms obtained from bevacizumab spiked plasma and plasma blank were compared. There was no any interference in six tested plasma blank.

3.1.3.2.2 Linearity, limits of detection (LOD), limits of quantification (LOQ)

Bevacizumab-spiked plasma samples were used to validate the established protocol. The linear range for quantification of bevacizumab in human plasma was designed according to the therapeutic range. Method linearity was evaluated from 30 μg mL^{-1} to 1000 μg mL^{-1}. The calibration curve was obtained with a weighting factor of 1/X and by linear regression analysis. The coefficient of determination was greater than 0.99, and the equation of this calibration curve is y=0.003460X-0.039664.The LOD and LOQ were 4 μg mL^{-1} and 10 μg mL^{-1} in plasma samples, respectively.

3.1.3.2.3 Accuracy and precision

The quantification accuracy, repeatability and intermediate precision were all evaluated at three concentrations. Accuracy evaluation used plasma samples obtained

doi:10.6342/NTU201804105

from both healthy volunteers and patients with infection diseases. Previous study has indicated immunoglobulin levels will be increased in infection conditions.[116] Considering the possible physiological variation of endogenous IgG which may affect accuracy of bevacizumab quantification, we used plasma samples from both healthy volunteers and patients with infection disease for evaluation of method accuracy. The accuracy was tested by spiking plasma samples with bevacizumab standard at three concentration levels and was expressed as percentage recovery. The overall percentage recoveries of three concentration levels were within 92.8 ± 3.2% to 112.7 ± 4.5%. Repeatability and intermediate precision in terms of peak area ratios (surrogate peptide/IS) at three concentration levels were lower than 5.2 and 12.9% RSD, respectively. Accuracy and precision test results are shown in Table 3.1.2.

3.1.3.2.4 Stability, matrix effect and extraction recovery

The previous study indicated that bevacizumab was stable in plasma samples at -20 °C for 15 days.[108] Process stability was evaluated in this study. Processed bevacizumab spiked plasma samples were placed in autosampler for 24 hours at 4 °C. The results indicated that all surrogate peptide signals were stable (RSD < 3%) after 24 hours storage at 4 °C for three tested concentrations. The matrix effects were tested at three concentration levels, and the results were between 94% and 125%. The extraction

doi:10.6342/NTU201804105

recoveries of protein G trapping procedure were tested at three concentration levels, and the recoveries were within 83.6 to 98.8%.

3.1.3.3 Clinical sample analysis

To evaluate the applicability of the established protocol, we applied it to quantify five plasma samples that were obtained from patients who were under bevacizumab treatment due to brain metastases of breast cancer. These patients all received bevacizumab at a dose of 15 mg kg^{-1}. Figure 3.1.6 shows one representative chromatogram from the analysis of one patients' plasma sample. The concentrations of bevacizumab and the clinical characteristics of the 5 patients are listed in Table 3.1.3 These results indicate that the method is effective for the quantification of bevacizumab in patient plasma samples.

3.1.3.4 Discussion

This study used protein G trapping and in-solution digestion coupled with LC-MS/MS to quantify bevacizumab in human plasma samples. ELISA is a commonly used analytical method for mAb quantification. Although it shows advantage in high throughput analysis, cross reactivity caused quantification problem was frequently being discussed.[102-103, 117-118] The amino acid sequence of humanized antibodies is 93-

doi:10.6342/NTU201804105

95% human. Since they show highly structure similar to endogenous IgGs, the endogenous IgGs may be possible interfere the results of ELISA method.[118-119] Compared to the ELISA based method, one of the most critical advantages of LC-MS/MS platform is that LC-MS/MS can provide better selectivity. To render the established LC-MS/MS method more applicable and economic in the clinical laboratory for therapeutic drug monitoring, protein G beads were used for sample purification. Compared to other antibody-based purification methods, the protein G method does not require specific antibodies to purify target proteins. Additionally, the cost for protein G purification is much lower compared to using customized antibodies. Although the protein G method is less selective, our study results revealed that IgG variations in each individual had a minimal effect on quantification accuracy.

To provide accurate quantification results, a suitable internal standard is necessary to calibrate possible variations that occur during sample preparation. Previous studies adopted extended SIL-peptides as the internal standards. However, one limitation of this method is that the digestion efficiency between the extended SIL-peptide and a monoclonal antibody may be different; as a result, extended SIL-peptides may not accurately mimic variations in the digestion procedure. The other limitation is that this extended SIL-peptide cannot correct for the fluctuation in the protein purification step, such as with protein G trapping used in this study. This finding is because the extended

doi:10.6342/NTU201804105

SIL-peptide cannot be trapped by the protein G beads. Compared to the extended SIL-peptide, a mAb-based internal standard with greater structural similarity to bevacizumab would provide better calibration performance. [120] Considering that it is too expensive to synthesize an SIL-mAb, we use tocilizumab as an internal standard. As the results show in Figure 3.1.5, tocilizumab successfully corrected the variations from the initial stage of protein trapping to the trypsin digestion, and thereby significantly improve the precision of the method. A similar concept has been proposed by Hongyan *et al*. In their study, they applied a mAb as a common IS in preclinical studies as this mAb does not exist in preclinical species but has peptide sequences that are common with target analytes. This common IS method should select general IgG sequences as their quantification surrogate peptides. However, as the majority of developed therapeutic mAbs are humanized IgGs, these general sequences also appear in endogenous IgGs. Therefore, their method is limited to pre-clinical studies and not for human samples. In contrast to their common IS, using tocilizumab as the IS is applicable to human sample analysis and provides good calibration performance at low cost.

Finally, the application to actual samples revealed that bevacizumab concentrations fluctuated significantly between individuals. The concentration difference may reach two-fold, even though they received the same dose. One phase I

doi:10.6342/NTU201804105

trial has reported bevacizumab concentration in plasma samples after drug administration at 15 mg kg^{-1}. The bevacizumab concentrations were ranged from 100 μg mL^{-1} to 400 μg mL^{-1} from day 0 to day 14.[121] In our study, the plasma sample was taken at day 1 after drug administration, and our detected concentration levels were similar with their reported results. However, that phase I study did not discuss the concentration fluctuation between each test individuals. Several recent studies indicated that the efficacies vary in samples from patients undergoing bevacizumab treatment.[117, 122-123] The other concern with the use of bevacizumab is side effects. Higher concentrations of bevacizumab may lead to side effects such as hemorrhage and phlebitis.[124] Therefore, a wider application of TDM for bevacizumab treatment could improve the therapeutic efficacy and reduce the side effects. The protocol proposed in this study, including protein G trapping and in-solution digestion with tocilizumab calibration, provided an effective, economic and readily assessable strategy for clinical laboratories to conduct TDM of bevacizumab to achieve personalized therapy.

3.1.4 Conclusions

In this study, we developed an LC-MS/MS method to quantify bevacizumab in human plasma using protein G trapping and in-solution digestion for sample

doi:10.6342/NTU201804105

pretreatment. We selected readily available and economical materials for sample preparation to facilitate its wider use in clinical fields. Protein G was used to trap the target analyte (bevacizumab) and minimize sample complexity. The IgG-based drug-IS tocilizumab exhibited good calibration performance. The validation results demonstrated that the method is accurate and could be used for pharmacokinetic studies and therapeutic drug monitoring for bevacizumab. Currently, the majority of available mAb drugs in the clinical field belong to the IgG class, and some IgG4 antibody drugs are currently undergoing clinical trials.[125] As the IgG class of drugs is now gaining more attention in the clinic, the general and simple protocol presented here may be applicable to other IgG class mAb drugs to improve the safety and effective use of mAb drugs.

doi:10.6342/NTU201804105

Table 3.1.1 MRM ion transitions and mass parameters for the surrogate peptides, SIL-IS and tocilizumab.

Amino acid sequence	MRM Transition	Collision energy	Fragmentor	Cell Accelerator voltage
VLIYFTSSLHSGVPSR[a]	588.3 → 602.3	35	150	7
FTFSLDTSK[b]	523.3 → 797.4	20	150	7
V*LIYFTSSLHSGVPSR	590.3 → 602.3	30	150	7
LLIYYTSR (Tocilizumab)	514.8 → 526.3	25	150	7

[a.] Used for quantification.

[b.] Used for confirmation.

[*.] Val(^{13}C5,^{15}N).

doi:10.6342/NTU201804105

Table 3.1.2. Accuracy and precision of bevacizumab quantification in plasma samples at three concentrations.

Concentration ($\mu g\ mL^{-1}$)	Accuracy (%Recovery)	Precision	
		Repeatability (%CV)	Intermediate precision (%CV)
30	112.7 ± 4.5	5.2	12.9
300	92.8 ± 3.2	1.2	3.2
1000	106.8 ± 4.2	2.7	9.5

doi:10.6342/NTU201804105

Table 3.1.3 The concentrations of bevacizumab and clinical characteristics of 5 breast cancer patients with brain metastases.

Number	Age	Sex	Height (cm)	Weight (kg)	Dosage	Disease status	Bevacizumab ($\mu g\ mL^{-1}$)
1	53	F	157	38	15 mg kg^{-1}	IV	345.2 ± 20.5
2	65	F	145	52.5	15 mg kg^{-1}	IV	468.6 ± 3.0
3	35	F	159	58	15 mg kg^{-1}	IV	651.7 ± 3.0
4	64	F	153	48.6	15 mg kg^{-1}	IV	302.4 ± 9.6
5	63	F	152	51	15 mg kg^{-1}	IV	352.2 ± 15.7

doi:10.6342/NTU201804105

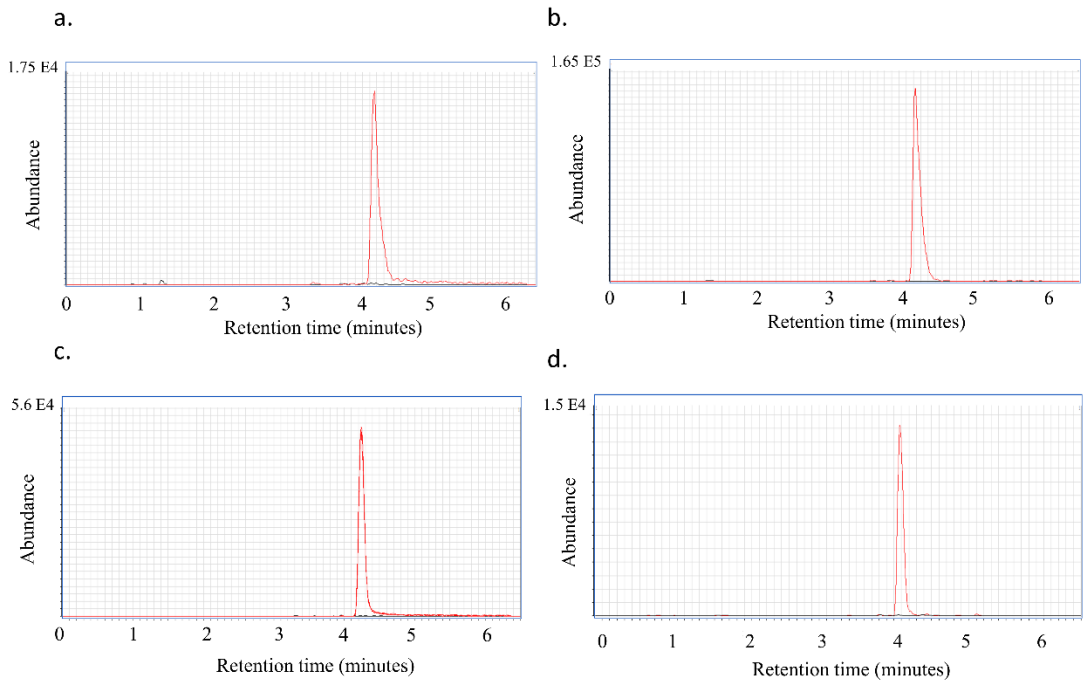


Figure 3.1.1 Overlay of MRM chromatograms of surrogate peptides from spiked and blank plasma samples. (a) and (b) are two surrogate peptides of bevacizumab: VLIYFTSSLHSGVPSR, 588.3→602.3 and FTFSLDTSK, 523.3→797.4. (c) is a surrogate peptide of SIL-IS, V*LIYFTSSLHSGVPSR, 590.3→602.3. (d) is a surrogate peptide of tocilizumab, LLIYYTSR, 514.8→526.3. The red color indicates a spiked plasma sample, and the black color indicates a plasma blank.

doi:10.6342/NTU201804105

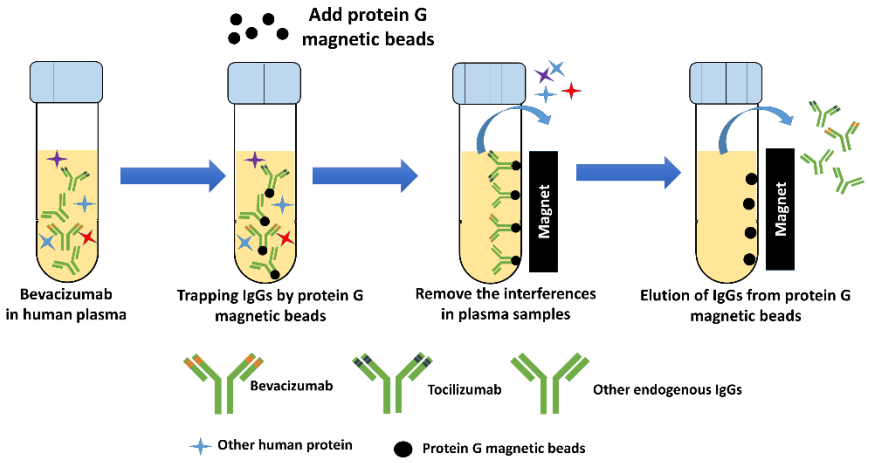


Figure 3.1.2 Schematic illustrations for protein G trapping of bevacizumab from human plasma.

doi:10.6342/NTU201804105

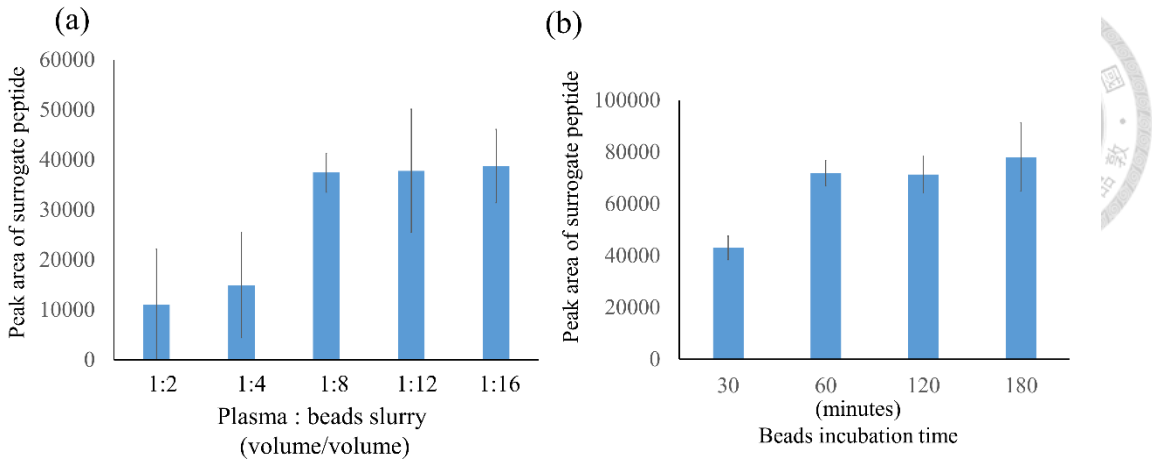


Figure 3.1.3 Optimization of the protein G purification procedure. (a) Effect of bead volume on signal intensity of a surrogate peptide of bevacizumab. Five microliters of plasma was mixed with different volumes of beads in five different groups. (b) Effect of bead incubation time on signal intensity of a surrogate peptide of bevacizumab. Five microliters of plasma, 40 μL of bead solution and 420 μL of PBS were mixed to evaluate incubation time.

doi:10.6342/NTU201804105

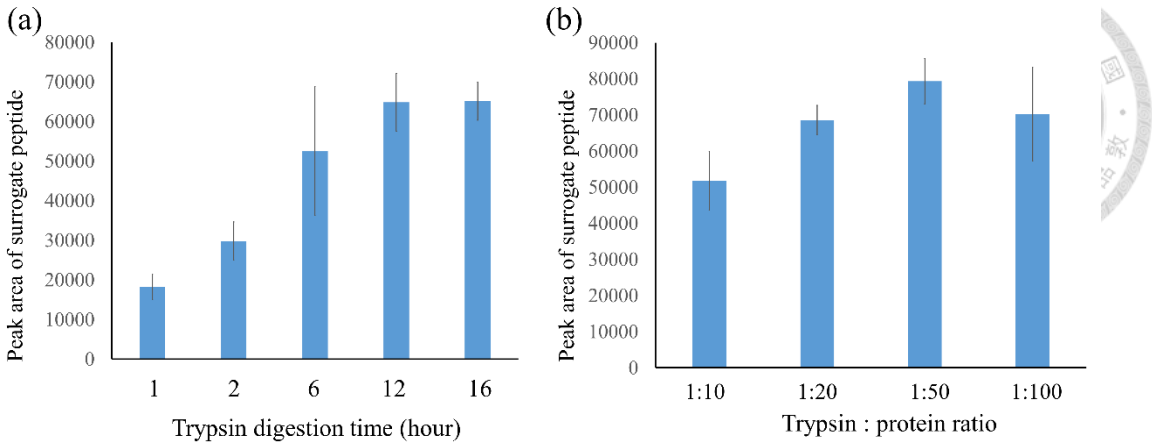


Figure 3.1.4 Optimization of the trypsin digestion procedure. Effect of (a) trypsin digestion time and (b) trypsin amount on the signal intensity of a surrogate peptide of bevacizumab.

doi:10.6342/NTU201804105

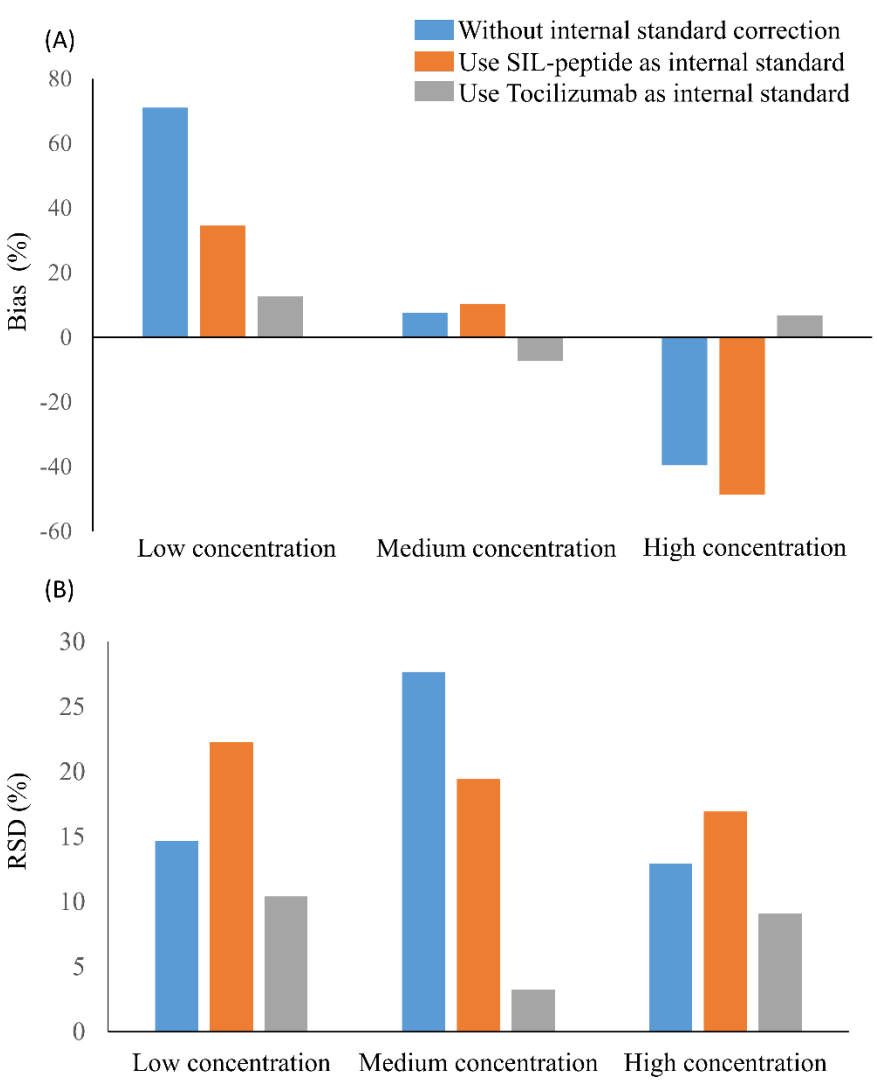


Figure 3.1.5 (A) Accuracy and (B) precision for three concentrations of QC samples with or without internal standard correction. The accuracy and precision with internal standard corrections was calculated based on two types of internal standards, including SIL-peptide and tocilizumab.

doi:10.6342/NTU201804105

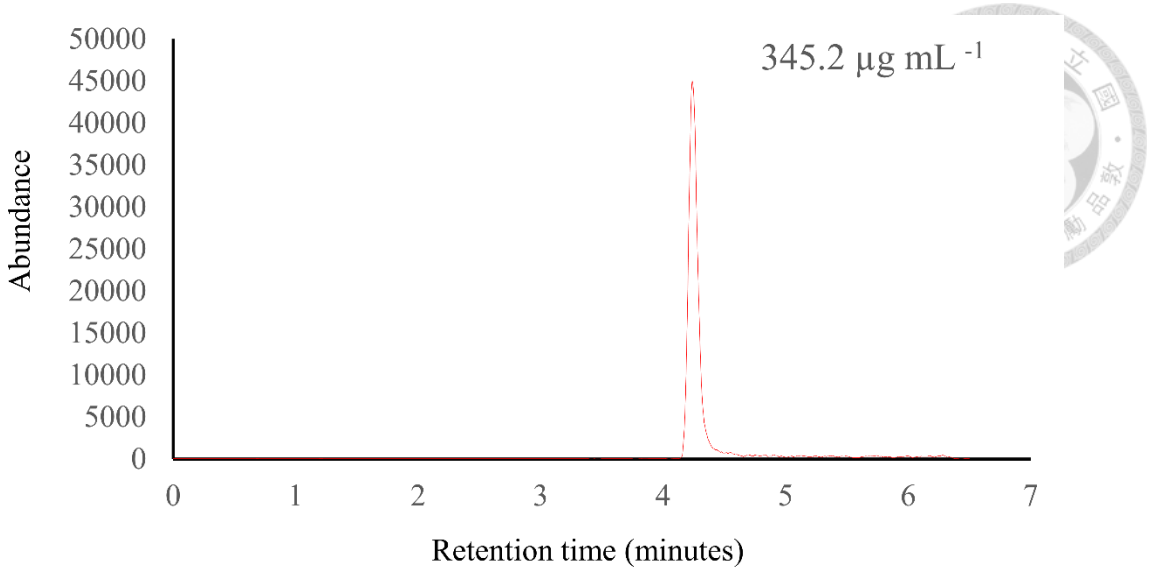


Figure 3.1.6 A representative chromatogram of a plasma sample obtained from a breast cancer patient with brain metastases. The bevacizumab dose was 15 mg kg^{-1}.

doi:10.6342/NTU201804105

Ch3.2 A general method for quantifying IgG-based therapeutic monoclonal antibodies in human plasma using protein G purification coupled with a two internal standard calibration strategy using LC-MS/MS

doi:10.6342/NTU201804105

3.2.1 Introduction

Recently, the development of therapeutic monoclonal antibodies (mAbs) has grown rapidly, with approval rates for new products in the US and Europe of 3~5 per year.[60] MAbs are used for several indications, including numerous types of cancers, and for inflammatory and immune diseases.[126-131] However, for many mAb drugs, the relationship between the optimal dose, efficacy and side effects are still unknown, and clinical outcomes vary among patients. In addition, due to the promising therapeutic efficacy of several mAb drugs, numerous drug combination strategies are in clinical trials, but the optimal dose for these combination therapies remains unclear.[132] Many pharmacokinetic/pharmacodynamic (PK/PD) studies are ongoing to establish exposure-response relationships for these combination therapies.

Precision medicine has generated much attention in recent years. To improve the efficacy and reduce the side effects, therapeutic drug monitoring (TDM) for dose adjustment has been recommended for several mAb drugs.[93, 130, 133-134] To perform TDM and to facilitate PK/PD studies with mAb drugs, an accurate, reliable and simple quantification method is an essential requirement. Conventional methods for measuring mAbs in biological fluids are based mainly on enzyme-linked immunosorbent assays (ELISAs).[96-97] However, ELISAs, having numerous limitations, such as an insufficient dynamic range, cross-reactivity, and the high cost and amount of time required for the

doi:10.6342/NTU201804105

production of specific antibodies, are not always the best choice for the measurement of mAbs.[99-103] Liquid chromatography–mass spectrometry (LC-MS)-based methods for the quantification of mAbs in biological fluids have recently become widely accepted because of their high sensitivity and selectivity.[115, 135-137]

A major concern with using LC-MS to measure mAb concentrations in biological fluids is that the complexity of the sample matrix may produce serious matrix effects (ME) during LC-MS analysis. To avoid matrix effects that lead to inaccurate quantification results, it is important to perform sample pretreatment and to correct MEs that occur with LC-MS analysis. Several sample preparation strategies have been proposed for mAb analysis by LC-MS.[109, 113, 138-139] However, these methods require specialized kits or specific antigens for analysis of mAbs. The most common approach used to correct MEs in LC-MS analysis is the inclusion of a stable isotope-labeled internal standard (SIL-IS). However, a specific SIL-IS is required for each mAb to be tested, and if commercially available, are usually extremely expensive.

Considering that an increasing number of mAb drugs are being used in clinical practice, a general analytical method for use in the clinical laboratory would facilitate clinical research and TDM for dose optimization. Currently, the majority of mAb drugs in clinical use are humanized IgGs. Because protein G is commonly used for IgG purification, we applied a general protein G purification strategy to trap target mAb

doi:10.6342/NTU201804105

drugs in human plasma samples.

Although protein G purification strategies are convenient and bind universally to IgG drugs, other endogenous IgG components in plasma may also be trapped by protein G that can produce matrix effects and quantification errors during LC-MS analysis. In a previous study, we proposed a post-column infused internal standard (PCI-IS) method to correct the matrix effects in LC-ESI-MS.[64] The main advantage of the PCI-IS method is that a single PCI-IS can be applied for correcting MEs of multiple analytes. Therefore, in this study, we used the PCI-IS method as a universal strategy to correct MEs that occur during the analysis of mAbs in LC-MS.

Three IgG-based drugs, including bevacizumab, nivolumab and pembrolizumab, were selected as demonstration drugs for optimization and evaluation of the general LC-MS method. Bevacizumab is a humanized anti-VEGF monoclonal IgG1 antibody, and was the first anti-angiogenesis agent to be approved by the Food and Drug Administration (FDA) for the treatment of metastatic colorectal cancer since 2004.[92] Nivolumab is a human IgG4 programmed cell death-1 (PD-1) immune checkpoint inhibitor antibody and is used for treatment of non-small cell lung cancer (NSCLC).[140] Pembrolizumab is a humanized monoclonal IgG4 antibody that targets PD-1 receptors and has been used for the treatment of metastatic melanoma and NSCLC.[141-142] Due to their promising therapeutic efficacy, various new indications and combination therapies

doi:10.6342/NTU201804105

are in clinical trials. Effective concentration measurement methods could facilitate these clinical studies and the treatment of patients. Finally, the proposed strategy was applied to analyze clinical samples obtained from cancer patients to demonstrate the applicability and potential of this general method in clinical analysis.

3.2.2 Materials and methods

3.2.2.1 Chemicals

Bevacizumab was purchased from Roche Applied Science (Indianapolis, IN, USA). Tocilizumab was purchased from Genentech (South San Francisco, CA, USA). Nivolumab was purchased from Ono Pharmaceutical Co., Ltd. (Japan). Pembrolizumab was purchased from Merck (Darmstadt, Hesse, Germany). Trypsin was purchased from Promega (Madison, WI, USA). Protein G Mag Sepharose Xtra beads were purchased from GE (Piscataway, NJ, USA). MS-grade methanol was purchased from Scharlau Chemie (Sentmenat, Barcelona, Spain). Acetonitrile (ACN), sodium chloride and sodium phosphate dibasic anhydrous were obtained from J.T. Baker (Phillipsburg, NJ, USA). Ammonium bicarbonate, formic acid (FA) solution (99%), dithiothreitol (DTT), iodoacetamide (IAA) and potassium phosphate monobasic were obtained from Sigma (St. Louis, MO, USA). Potassium chloride was purchased from Fluka (Buchs, St.

doi:10.6342/NTU201804105

Gallen, Switzerland). The extended SIL-IS was synthesized by GenScript (Piscataway, NJ, USA).

3.2.2.2 LC-MS/MS

An Agilent 1290 UHPLC equipped with an Agilent 6460 triple quadrupole system (Agilent Technologies, Waldbronn, Germany) was used for the analyses. An Aeris™ PEPTIDE XB-C18 100 x 2.1 mm (1.7 μm) column (Phenomenex, Torrance, USA) was selected for the separation. The mobile phase consisted of 0.1% aqueous formic acid (solvent A) and 0.1% formic acid in ACN (solvent B) at a flow rate of 0.3 mL min^{-1}. The gradient profile was as follows: 0-1 min, 10% B; 1-7.5 min, 10-32% B; 7.5-7.7 min, 32-100% B; 7.7-9.5 min, 100% B; and the column was re-equilibrated with 10% B for 1.5 min. The sample reservoir was maintained at 4 °C, and the column oven was set at 40 °C. The injection volume was 18 μL. A positive electrospray ionization mode was utilized with the following parameters: a dry gas temperature of 325 °C; a dry gas flow rate of 7 L min^{-1}; a nebulizer pressure of 45 psi, a sheath gas temperature of 325 °C; a sheath gas flow rate of 11 L min^{-1}; a capillary voltage of 3500 V and a nozzle voltage of 500 V. MS acquisition was conducted in multiple reaction monitoring (MRM) mode. Post column infused internal standard (PCI-IS) was dissolved in 0.1% FA in 50% methanol and 50% deionized water at 500 ng mL^{-1} and introduced into the

doi:10.6342/NTU201804105

MS at a flow rate of 0.2 mL min^{-1}.

The transitions for surrogate peptides of bevacizumab, pembrolizumab and nivolumab were as follows: m/z 588.3→602.3, 553.4→667.4 and 550.6→661.5, respectively. The transition for the internal standard tocilizumab was 514.8→526.25, and the transition for the post column infused internal standard was 557.6→678.1.

3.2.2.3 Trapping and digestion of mAb drugs from human plasma

First, protein G beads were used to trap the target therapeutic mAb drugs from human plasma. Eighty microliters of protein G magnetic bead solution was conditioned twice with 200 μL of phosphate-buffered saline (PBS). Next, 10 μL of plasma and 20 μL of tocilizumab (50 μg mL^{-1}) were added to the bead solution. After incubating for 1 hour at 4 °C, 200 μL of PBS buffer and 200 μL of deionized water were used sequentially as washing solutions to remove unbound proteins. The 3 mAb drugs were eluted from the beads with two applications of 200 μL of 100 mM formic acid solution. The eluent was collected and dried under N_2. The dried eluent was reconstituted with 200 μL of 50 mM ammonium bicarbonate buffer and the solution was heated at 90 °C for 25 min. Ten microliters of 100 mM DTT was added to the solution and then heated at 60 °C for 60 min. After cooling, 10 μL of 200 mM IAA was added for alkylation at 30 °C for 30 min in the dark. For digestion with trypsin, 36 μL of trypsin (0.1μg $μL^{-1}$)

doi:10.6342/NTU201804105

was added to the solution and heated at 37 °C for 14 hours. After 14 hours of digestion, 20 μL of 10% FA was added to the trypsin digestion solution to terminate the digestion. The digested sample was then subjected to LC-MS/MS analysis.

3.2.2.4 Calibration using two internal standards

The surrogate peptide signal intensities for each mAb and IS (tocilizumab) at every time point in the chromatogram were divided by the PCI-IS responses at the same retention times to correct for ME. The acquired ratios were then applied to generate the new adjusted chromatogram. Next, we integrated the peak areas of each surrogate peptide and internal standard (tocilizumab) in the adjusted chromatogram. After determining the peak areas of each surrogate peptide of the target analytes and the internal standard (tocilizumab), the ratio of the peak area of each surrogate peptide to internal standard (tocilizumab) was used for quantification

3.2.2.5 Data analysis

All of the MRM chromatograms that were obtained from Agilent Data Analysis software, including surrogate peptides of the three target mAbs, IS (tocilizumab) and PCI-IS, were collected and exported to.xls files. These data were further plotted using

doi:10.6342/NTU201804105

R programming software.[143] The final export peak area table was processed using Microsoft Excel 2007 (Microsoft Corp., Redmond, WA, USA).

3.2.2.6 Method validation

3.2.2.6.1 Selectivity

To evaluate method selectivity, six plasma blank samples (without addition of bevacizumab, pembrolizumab, nivolumab and tocilizumab) and three mAb-spiked samples were investigated and compared. The chromatograms of plasma blanks and three mAb-spiked samples were compared to confirm whether there was any interference at the same retention time.

3.2.2.6.2 Linearity, limits of detection (LOD) and limits of quantification (LOQ)

Each calibration curve was designed based on their potential therapeutic ranges. For bevacizumab, a series dilution of the stock solution (25 mg mL^{-1}) was performed, and each solution was added to plasma blanks to obtain 15, 30, 100, 200, 400 and 800 μg mL^{-1} spiked samples that were used to generate the calibration curve. For nivolumab,

doi:10.6342/NTU201804105

a series dilution of the stock solution (10 mg mL^{-1}) was conducted, and each solution was added to plasma blanks to obtain 10, 40, 100, 200, 400 and 800 μg mL^{-1} spiked samples that were used to generate the calibration curve. For pembrolizumab, a series dilution of the stock solution (25 mg mL^{-1}) was performed and each solution was added to plasma blanks to obtain 5, 10, 40, 200, 400 and 800 μg mL^{-1} spiked samples that were used to generate the calibration curve. Each concentration for the calibration curve was analyzed in triplicate. The peak areas of the analytes were integrated using R software. The calibration curves were obtained with a weighting factor of 1/X and by linear regression analysis. The limit of detection (LOD) was defined as a signal to noise (S/N) ratio of three. The limit of quantification (LOQ) was defined as a signal to noise (S/N) ratio of ten.

3.2.2.6.3 Accuracy and precision

To evaluate accuracy, three different concentrations of each mAb drug (15, 200 and 800 μg mL^{-1} in plasma for bevacizumab; 5, 200 and 800 μg mL^{-1} in plasma for pembrolizumab; and 10, 200 and 800 μg mL^{-1} in plasma for nivolumab) were spiked into the different plasma samples from healthy controls and patients with infection, and the back-calculated concentrations were compared to the spiked concentrations. The accuracies were determined using five determinations per concentration. For

doi:10.6342/NTU201804105

repeatability evaluations, the samples were analyzed in triplicate per concentration within the same day. For intermediate precision, three samples per concentration were prepared individually and analyzed on three different days.

3.2.2.6.4 Stability and matrix effects

The stability of processed samples was evaluated by comparing the results for samples analyzed immediately after preparation and those analyzed after storage in a 4 °C autosampler for 24 h. The stability was evaluated for each of the three mAb drugs at low, medium and high concentrations, and the results are presented as the recovery (%) relative to the samples analyzed immediately after preparation. To evaluate matrix effects, three concentrations of digested mAb standards were spiked individually into three plasma blank samples and one reagent blank sample. The reagent blank was composed of the same components that were present in each mAb digestion buffer. The matrix effects of the three mAb drugs at three concentrations were calculated using their own surrogate peptide intensities in plasma blanks divided by the surrogate peptide intensities in reagent blanks.

doi:10.6342/NTU201804105

3.2.2.7 Collection of clinical samples

Plasma samples were collected at the National Taiwan University Hospital. The study was approved by the institutional review board of the National Taiwan University Hospital (201709061RIND). Informed consent was obtained from all participants. Avastin® (bevacizumab) was administered intravenously at a dose of 15 mg kg^{-1}. Plasma samples were obtained from three patients who received bevacizumab after three weeks. OPDIVO® (nivolumab) was administered intravenously at a dose of 3 mg kg^{-1}. Plasma samples were obtained from two patients who received nivolumab after two weeks. Keytruda® (pembrolizumab) was administered intravenously at a dose of 1.8 mg kg^{-1}. Plasma samples were obtained from one patient who received pembrolizumab after 2 weeks. All the collected blood samples were centrifuged and the resultant plasma samples were stored at −80 °C until use.

3.2.3 Results and discussion

3.2.3.1 Method development

3.2.3.1.1 Selection of surrogate peptides for bevacizumab, nivolumab and

doi:10.6342/NTU201804105

pembrolizumab

Surrogate peptides for each mAb were used to quantify each mAb drug in plasma samples. Bevacizumab, pembrolizumab and nivolumab were spiked individually into plasma blank samples, and each sample was profiled by time-of-flight mass spectrometry to identify unique peptides that were contributed by the mAbs. The observed unique m/z was compared to the amino acid sequence of surrogate peptides using the website PeptideMass (http://web.expasy.org/peptide_mass/). Three surrogate peptides, VLIYFTSSLHSGVPSR, DLPLTFGGGTK and ASGITFSNSGMHWVR for bevacizumab, pembrolizumab, and nivolumab, respectively, were identified, and these peptides were examined further for their MRM transitions for quantification. The amino acid sequences, MRM transitions and detailed mass parameters for surrogate peptide analysis are listed in Table 3.2.1. The selectivity was verified by comparing the chromatograms obtained by mAb-spiked and plasma blanks. The results indicated that the selected surrogate peptides were selective for bevacizumab, pembrolizumab and nivolumab quantification in plasma samples (Figure 3.2.1).

3.2.3.1.2 Purification of mAb drugs using protein G beads

Protein G magnetic beads were used to universally trap IgG-based mAb drugs

doi:10.6342/NTU201804105

from plasma samples. Bevacizumab is an IgG1 antibody, and pembrolizumab and nivolumab are IgG4 antibodies. Protein G beads effectively trap these mAbs regardless of their IgG subtypes. The workflow for the sample preparation procedure is shown in Figure 3.2.2. Protein G magnetic beads were mixed with the mAb-spiked plasma samples. Endogenous IgG and target analytes were trapped by the magnetic beads because of the affinity between protein G and IgG. High levels of other interfering proteins such as albumin will be removed by a washing step. An acidic buffer was used to disrupt the interaction between protein G and the analytes. Figure 3.2.1 shows that the surrogate peptides for bevacizumab, pembrolizumab and nivolumab are all readily detected in the LC-MS chromatograms, indicating that protein G effectively traps these IgG drugs, thus reducing the sample matrix's complexity and improving the sensitivity. Moreover, this purification step can lead to lower consumption of trypsin, rendering this general method more economical. Using protein G magnetic beads to trap IgG-based mAb drugs represents an effective and convenient strategy for the universal purification of IgG-based mAb drugs from human plasma.

3.2.3.1.3 Optimization of the sample preparation procedure

Protein G beads were incubated with plasma samples that were spiked with three mAb drugs to determine the optimal incubation time. To effectively trap these mAb

doi:10.6342/NTU201804105

drugs, 30- to 150-minute incubation times were evaluated for their trapping efficiency. The buffer volume and manner of incubation were described in our previous report.[144] As the results show in Figure 3.2.3 A, the abundance of surrogate peptides increased from 30 to 60 minutes for all three mAb drugs; however, if the incubation time was increased further, the amount of surrogate peptide did not increase. The three mAb drugs exhibited similar profiles between the trapping time and the LC-MS signal abundance, and 60 minutes was selected as the optimal incubation time for the protein G purification step.

Trypsin digestion was performed after protein G purification to obtain surrogate peptides for LC/MS quantification. To ensure that the digestion was complete for each of the three demonstration mAbs, plasma samples that were spiked with the three mAbs were evaluated to investigate the efficiency of trypsin digestion. To acquire the optimal trypsin digestion performance, digestion times from 2 to 24 hours were evaluated. Considering the possibility that individual differences in plasma components may affect the digestion efficiency, three different plasma samples were used to explore the optimal digestion time. The results shown in Figure 3.2.3 B indicate that for digestion times from 2 to 14 hours, the abundance of surrogate peptides was clearly increased for all three demonstration mAb drugs; however, as the digestion time increased, there was no additional increase in the abundance of surrogate peptides. Therefore, 14 hours was

doi:10.6342/NTU201804105

selected as the optimal digestion time.

3.2.3.1.4 Use of two internal standards for improving quantification accuracy

To obtain high quantification accuracy, internal standards should be added to calibrate potential errors that are caused by sample preparation and LC-MS analysis. A SIL-IS can be used to calibrate potential errors that arise from both the sample preparation step and from LC-MS analysis, and it is the ideal internal standard for LC-MS quantification. However, each analyte requires its' own respective SIL-IS when using the SIL-IS calibration method, and it cannot be regarded as a universal internal standard. To establish a general LC-MS method for IgG-based therapeutic monoclonal antibody quantification, we adopted the PCI-IS method as a general strategy to evaluate and calibrate matrix effects that arise during LC-MS analysis. Previous study indicated that hydrophobicity and protonation ability are two important factors to consider for the selection of a PCI-IS.[64] The similar retention times for the three surrogate peptides reveal their similar hydrophobicity. Due to all surrogate peptides are built up by the amino acids, they should show similarity in protonation property. Therefore, peptide sequences with similar molecular weights to the three surrogate peptides should provide

doi:10.6342/NTU201804105

good ME corrections for these three surrogate peptides. In this study, we used an extended isotope-labeled surrogate peptide for bevacizumab as our PCI-IS. The chromatogram of the PCI-IS is shown in Figure 3.2.4, and the analyte signals were calibrated using signals obtained with the PCI-IS. Although the PCI-IS could address matrix effects that caused quantification errors, it cannot address potential errors from the sample preparation step. Therefore, a two internal standard approach was adapted in this method.

In addition to PCI-IS, an IgG-based drug internal standard was used to address the potential errors that arise in the sample preparation step, including any trapping loss from protein G and the fluctuation of trypsin digestion efficiency. Previous study has indicated that using the entire molecule as the internal standard can provide a better correction result compared to the extended peptide. [111] We have previously demonstrated that the use of IgG-based drugs can provide good correction in both protein G purification and trypsin digestion compared to the extended peptide. [144] Therefore, we selected the IgG-based drug tocilizumab as our internal standard to calibrate potential variations that arise during sample preparation steps. To perform a calibration using the two internal standard method, the LC-MS chromatograms were first corrected with the PCI-IS, and the signal intensities of each surrogate peptide were corrected by the signal intensity of the PCI-IS. The PCI-IS-corrected surrogate peptide

doi:10.6342/NTU201804105

signals were then calibrated using the tocilizumab signal intensity.

The correction performance by the two internal standards approach was evaluated at three concentrations using QC samples. The overall accuracies and precisions before and after calibration are summarized in Figure 3.2.5. Figure 3.2.5.A-C demonstrate that the accuracies were greater than 115% at low and high concentrations for certain mAb drugs. Using the two internal standard approach could effectively improve the accuracy of quantification, producing errors within 15% for all mAb drugs at three test concentrations. The improvement in method precision for the two internal standards method was evaluated by analyzing three samples that were obtained from different healthy volunteers, and the results are shown in Figure 3.2.5.D-E. The RSDs of three replicates were generally reduced after being calibrated by the two internal standard method, and the RSDs were all within 15.0% except for nivolumab at low concentrations. The results demonstrate that this two IS correction approach could effectively improve method accuracy and precision for the quantification of IgG-based drugs by LC-MS.

doi:10.6342/NTU201804105

3.2.3.2 Method validation

3.2.3.2.1 Selectivity

Selectivity was evaluated using six plasma samples that were obtained from healthy controls and patients. No interference was produced by any of the six tested plasma blanks.

3.2.3.2.2 Linearity, limits of detection (LOD) and limits of quantification (LOQ)

Three mAb-spiked plasma samples were used for method validation. The calibration curves were designed according to the therapeutic range of each mAb drug. Method linearity was evaluated from 15 to 800 μg mL^{-1} for bevacizumab, 10 to 800 μg mL^{-1} for nivolumab and 5 to 800 μg mL^{-1} for pembrolizumab. The coefficient of determination was greater than 0.99 for all three tested mAb drugs, and the equations of the calibration curves were y=0.006436X-0.00229, y=0.011963X-0.00256 and y=0.014455X-0.0061 for bevacizumab, nivolumab and pembrolizumab, respectively. The LOD was 4, 5, and 1 μg mL^{-1} in plasma samples for bevacizumab, nivolumab and pembrolizumab, respectively. The LOQ was 8, 8 and 3 μg mL^{-1} in plasma samples for

doi:10.6342/NTU201804105

bevacizumab, nivolumab and pembrolizumab, respectively.

3.2.3.2.3 Accuracy and precision

We evaluated the quantification accuracy, repeatability and intermediate precision at three concentrations for all three mAb drugs. Immunoglobulin levels are increased in infection and in several disease conditions. To consider the potential fluctuation in endogenous immunoglobulin levels and to evaluate the effect of the fluctuation on mAb drug analysis in plasma samples, we included plasma samples that were obtained from patients with infection diseases in accuracy and precision tests.[116] The accuracy was verified by spiking plasma samples with the 3 mAb drug standards at three concentration levels, and the recoveries were expressed as percentages. The overall percent recoveries at three concentration levels for the 3 mAb drugs were all within 89.2 ± 4.5% to 115.7 ± 2.7%. Repeatability and intermediate precision at three concentration levels for the 3 mAb drugs were less than 6.5 and 9.0% RSD, respectively. The results of accuracy and precision test are summarized in Table 3.2.2.

3.2.3.2.4 Stability and matrix effects

The process stabilities of three mAb drugs were evaluated in this study. Three

doi:10.6342/NTU201804105

mAb-spiked plasma samples were placed in an autosampler for 24 hours at 4 °C. The results obtained indicated that all surrogate peptides for each mAb drug were stable for 24 hours at 4 °C and that greater than 94.6 ± 3.4% recovery could be obtained for the three mAb-spiked samples at three tested concentrations. Three mAb-spiked samples were used to evaluate matrix effects at three concentrations. For nivolumab-spiked samples, the matrix effects at three concentrations were between 75% and 86%. For pembrolizumab-spiked samples, the matrix effects at three concentrations were between 77% and 82%. For bevacizumab spiked samples, the matrix effects at three concentrations were between 108% and 121%.

3.2.3.3 Clinical applications

The applicability of this general method was evaluated by 6 different plasma samples that were obtained from patients who were undergoing mAb drug treatment with nivolumab, bevacizumab or pembrolizumab. For all clinical samples, patients received different mAb treatments at day 1. For patients who received nivolumab and pembrolizumab treatments, the samples were collected 14 days after drug administration. For patients who received bevacizumab treatment, the samples were collected 21 days after drug administration. The dosage of each mAb is listed in Table 3.2.3. Plasma samples were collected at trough levels to ensure the method sensitivity

doi:10.6342/NTU201804105

could cover the entire therapeutic cycle. The quantification results and the clinical characteristics of the 6 patients are also displayed in Table 3.2.3. The results demonstrate that the general method provided sufficient sensitivity for quantifying the 3 demonstration mAb drugs in human plasma.

3.2.3.4 Discussion

To facilitate personalized treatments with mAb drugs and to better understand their PK/PD properties in various ongoing trails, the development of a general method to quantify the therapeutic mAbs concentrations in human plasma is very important. ELISAs are currently the most commonly used analytical tool for mAb quantification, but they have several limitations and are specific for one particular drug per ELISA kit. [102-103, 117-118] Compared to ELISA methods, LC-MS/MS has the advantage of minimizing cross reactivity problems and provides an opportunity to establish a general method to quantify different mAb drugs, which is especially important in clinical laboratories.

To provide a general and reliable LC-MS/MS method for quantifying different therapeutic mAb drugs, considerations of both purification strategies and correction of matrix effects in LC-MS are critical issues. In this study, we developed a general method that combined an immuno-affinity strategy and a two internal standard

doi:10.6342/NTU201804105

correction approach for quantifying IgG-based therapeutic monoclonal antibodies in human plasma. Three drugs belonging to IgG 1 and 4 subclasses were selected as the demonstration drugs.

For the sample preparation step, we used protein G beads to remove interfering proteins followed by trypsin digestion for peptide analysis by LC-MS. We observed a similar trend in the optimization of the protein G purification time and the trypsin digestion time for the three demonstration mAb drugs, and it is anticipated that the optimal sample preparation procedure could be applied to other IgG drugs.

Although the three demonstration drugs exhibited similar trends in the optimization of trapping and digestion times, individual variations in trapping recovery and digestion efficiency may still occur when applying the optimal sample preparation procedure. We therefore used another IgG drug, tocilizumab, as the ISTD to address any potential bias that might arise during the sample preparation step. However, the use of tocilizumab as an ISTD was limited because its' retention time differed from the test drugs and could not provide excellent calibration of matrix effects in LC-MS analysis. To correct matrix effects that occur in LC-MS, the PCI-IS was used to calibrate multiple analytes using single ISTD. Our results demonstrate that this two internal standard correction method could significantly improve quantification accuracy and method precision. Because this approach does not require the use of a specific antibody or

doi:10.6342/NTU201804105

synthesis of its' own SIL –mAb for each therapeutic mAb drug for sample preparation and ME correction, it possesses advantages of economy and convenience. IgG class mAb drugs represent a high percentage of drugs that have been recently approved by the FDA, and several IgG-based drugs, including romosozumab, avelumab and durvalumab may obtain approvals in 2017.[145] It is believed that this general method has the potential to perform TDM or to investigate the PK/PD properties for these IgG-based therapeutic mAb drugs.

3.2.4 Conclusions

In this study, we developed a general LC-MS/MS method that combines a two internal standard correction approach to quantify three therapeutic mAb drugs in human plasma. Protein G bead purification was selected to minimize sample complexity. An IgG-based drug-IS, tocilizumab, and a PCI-IS were utilized to improve quantification accuracy. The validation results demonstrated that this general method is accurate and precise, and its application in clinical analysis revealed that the method is adequately sensitive for therapeutic monitoring. It is believed that this general method could be applied to other IgG-based mAb drugs, which would be a benefit to precision medicine and could facilitate various clinical studies.

doi:10.6342/NTU201804105

Table 3.2.1 MRM ion transitions and mass parameters for the surrogate peptides of bevacizumab, pembrolizumab, nivolumab, and the two internal standards.

analyte name	Amino acid sequence	MRM Transition	Collision energy (eV)	Fragmentor (V)	Cell Accelerator voltage (V)
Bevacizumab	VLIYFTSSLHSGVPSR	588.3 → 602.3	35	150	7
Pembrolizumab	DLPLTFGGGTK	553.4 → 667.4	25	135	7
Nivolumab	ASGITFSNSGMHWVR	550.6 → 661.5	20	150	7
Internal standard (Tocilizumab)	LLIYYTSR	514.8 → 526.3	25	150	7
Post-column infused internal standard	PGKAPKV*LIYFTSSLHSGVPSRFSGSG	557.6 → 678.1	10	100	7

* : Val(^{13}C5,^{15}N)

doi:10.6342/NTU201804105

Table 3.2.2 Accuracy and precision of bevacizumab, pembrolizumab and nivolumab quantification in plasma samples at three concentrations.

Therapeutic mAb	Accuracy (% Recovery)			Precision					
				Repeatability (% CV)			Intermediate precision (% CV)		
	Low QC	Medium QC	High QC	Low QC	Medium QC	High QC	Low QC	Medium QC	High QC
Bevacizumab	97.1 ± 4.1	97.6 ± 8.8	112.4 ± 7.6	3.5	3.3	4.3	4.2	9.0	6.8
Pembrolizumab	91.9 ± 6.5	100.0 ± 2.1	115.7 ± 2.7	6.5	3.4	4.3	7.1	2.1	2.4
Nivolumab	93.3 ± 5.8	89.2 ± 4.5	103.1 ± 6.1	5.7	2.5	4.4	6.2	5.0	5.9

doi:10.6342/NTU201804105

Table 3.2.3 The concentrations of bevacizumab, nivolumab, pembrolizumab and clinical characteristics of 6 patients.

Administrated mAb drug	Dose (mg kg^{-1})	Cancer type	Gender	Age	Measured concentration (μg mL^{-1})
Bevacizumab	15	breast cancer	F	56	20.1
	15	breast cancer	F	56	87.2
	15	breast cancer	F	48	75.1
Nivolumab	3	hepatocellular carcinoma	F	69	44.2
	3	hepatocellular carcinoma	M	49	51.3
Pembrolizumab	1.8	breast cancer	F	33	10.3

doi:10.6342/NTU201804105

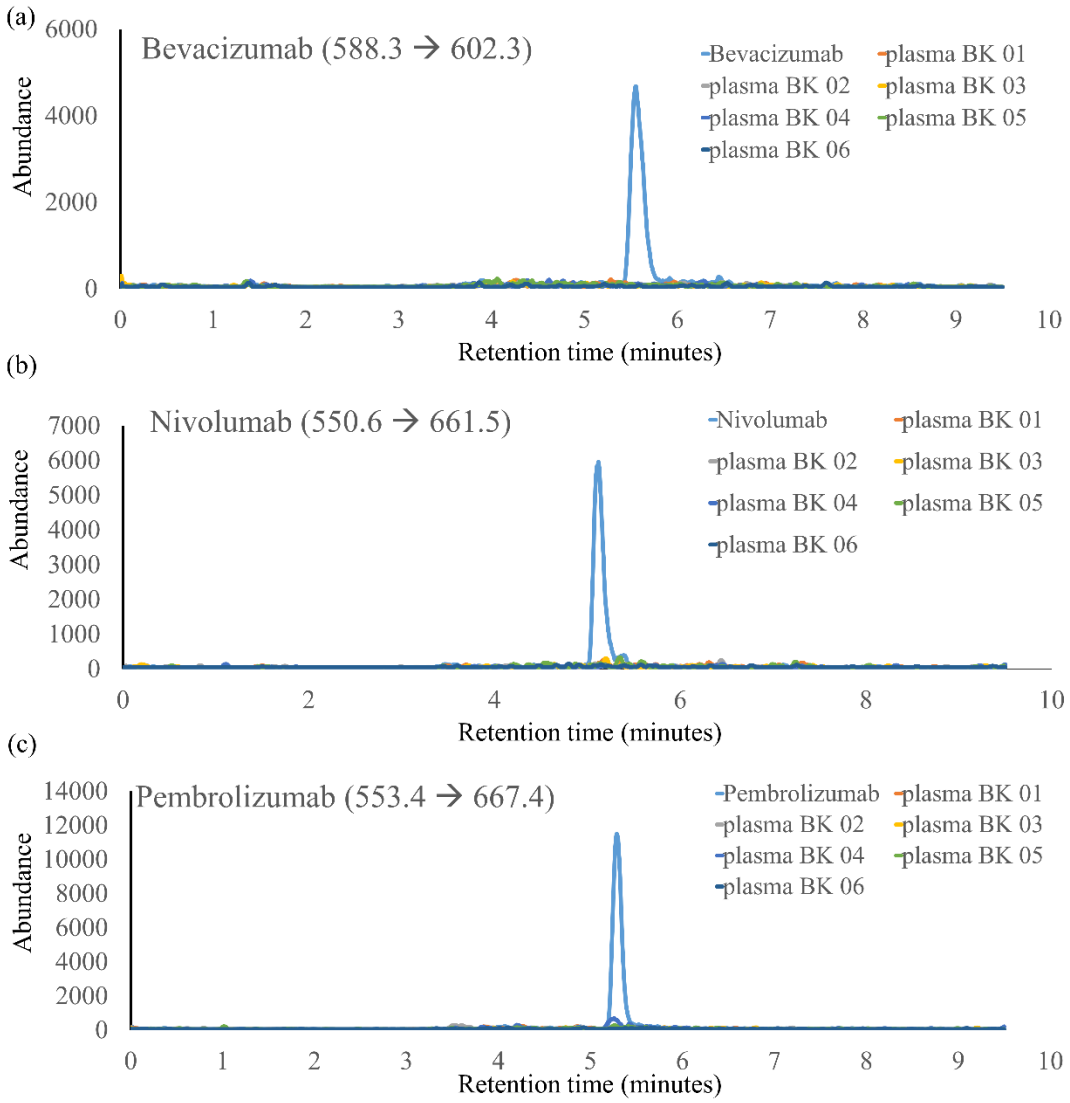


Figure 3.2.1 Overlay of MRM chromatograms of surrogate peptides from spiked and blank plasma samples. (A) is the surrogate peptide for bevacizumab: VLIYFTSSLHSGVPSR, 588.3→602.3, (B) is the surrogate peptide for nivolumab: ASGITFSNSGMHWVR, 550.6 → 661.5, and (C) is the surrogate peptide for pembrolizumab: DLPLTFGGGTK, 553.4→667.4. The light blue color indicates a spiked plasma sample, and the other colors indicate six plasma blank samples.

doi:10.6342/NTU201804105

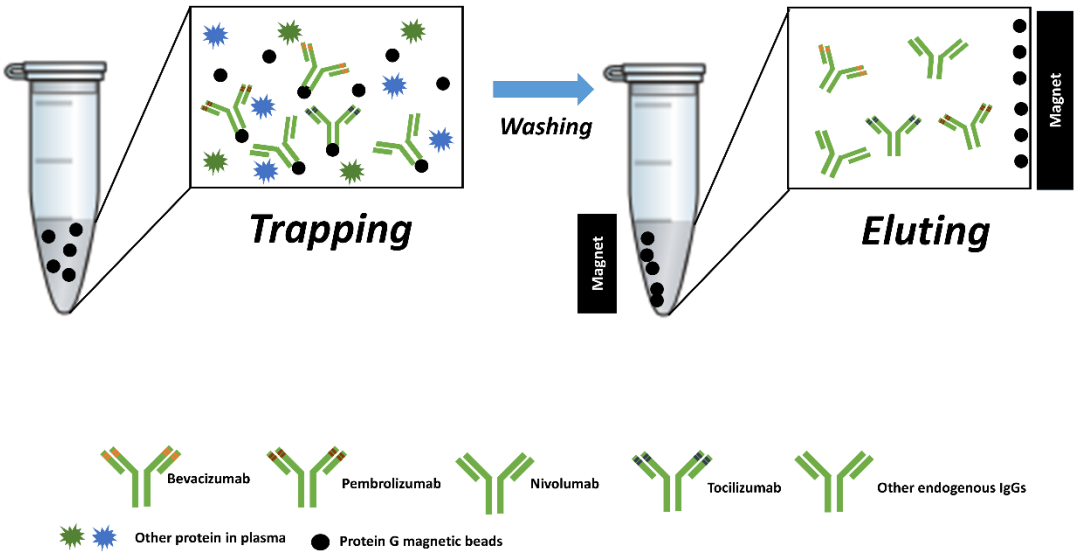


Figure 3.2.2 Schematic illustration of protein G purification of three mAb drugs (bevacizumab, nivolumab, and pembrolizumab) and the internal standard (tocilizumab) from human plasma.

doi:10.6342/NTU201804105

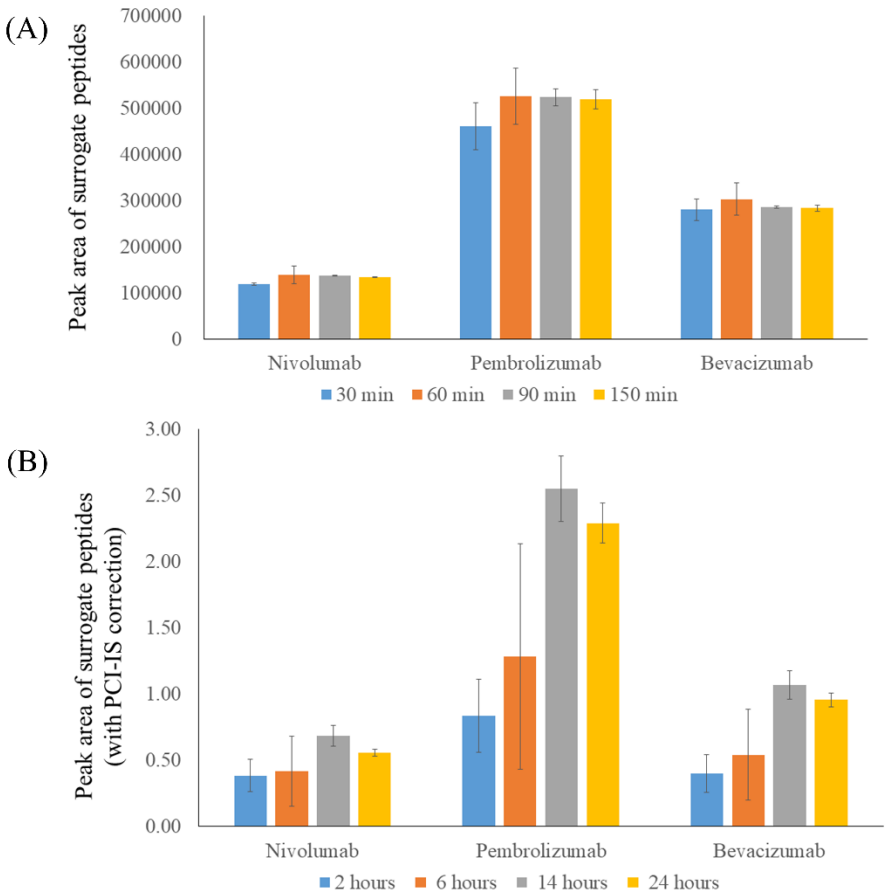


Figure 3.2.3 Optimization of the sample preparation procedure. Effect of (A) protein G purification time and (B) trypsin digestion on the signal intensities of surrogate peptides of bevacizumab, nivolumab and pembrolizumab.

doi:10.6342/NTU201804105

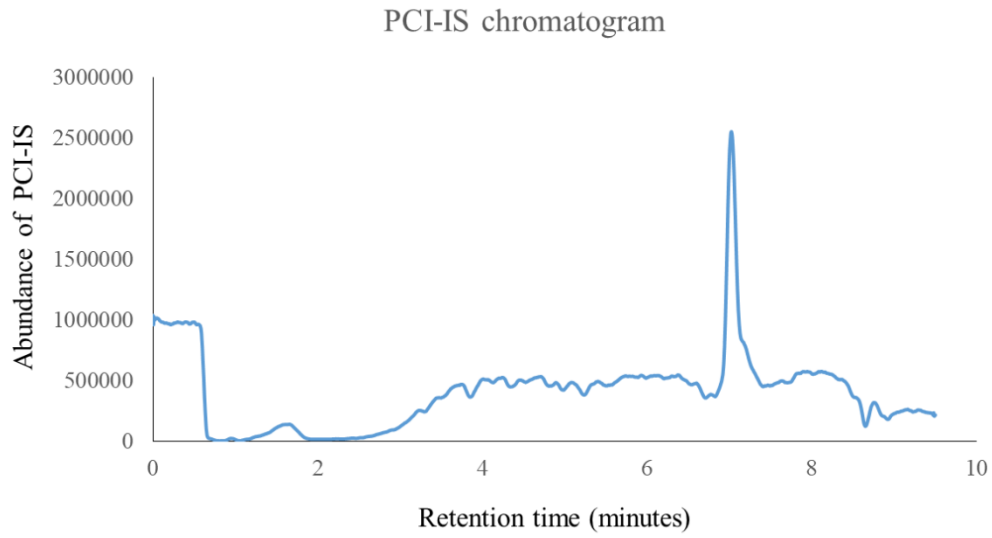


Figure 3.2.4 MRM chromatogram of PCI-IS. (MRM transition: 557.6→678.

doi:10.6342/NTU201804105

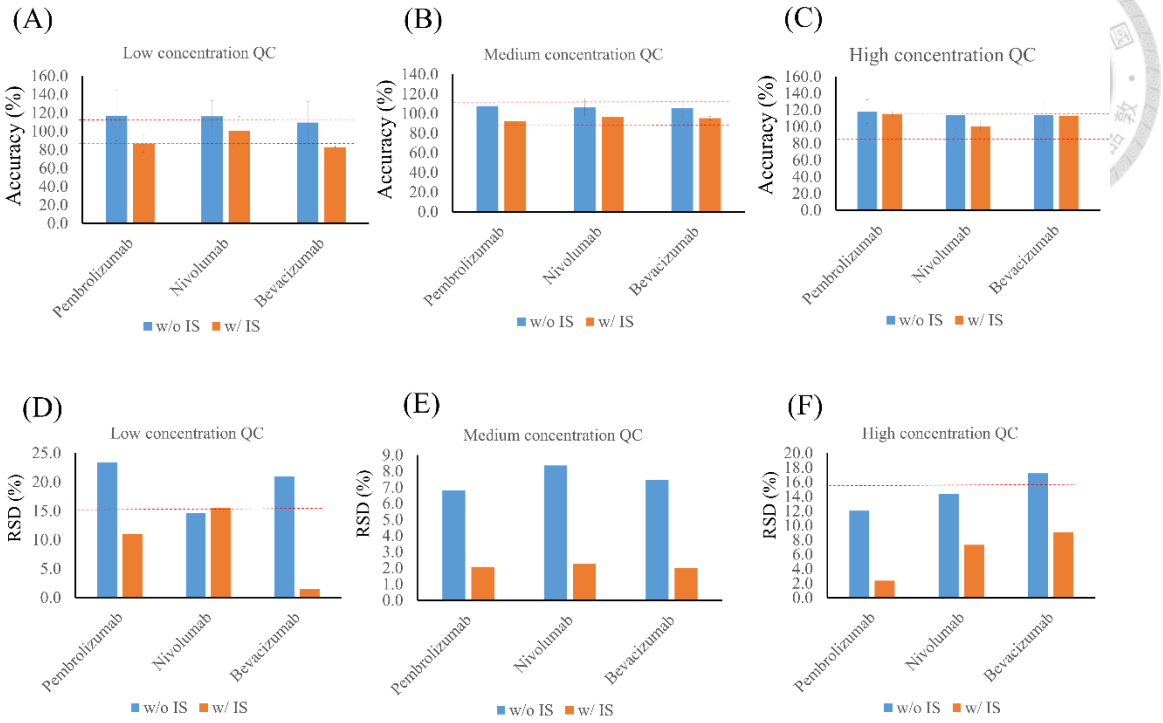


Figure 3.2.5 Comparison of accuracy and precision obtained with and without two internal standards calibration. (A) to (C) are accuracy comparisons and (D) to (F) are precision comparisons for three concentrations of QC samples. The blue color indicates the results without the two internal standard correction method, and the red color indicates the results with the two internal standard correction method.

doi:10.6342/NTU201804105

Chapter 4. Summary and Perspectives

doi:10.6342/NTU201804105

4.1 Summary and perspective

As the cancer incident rate is increasing, it is important to have effective methods for cancer prevention, early diagnosis, and treatment. This thesis mainly focused on two major issues: (1) using targeted metabolomics to quantify fatty acids and to investigate the potential fatty acid markers for breast cancer (BC) detection (chapter 2.) and (2) using protein G purification combine with LC-MS/MS to quantify mAb drugs in human plasma samples. (chapter 3.)

To improve the outcomes of cancer therapy and reduce the cancer mortality, it is important to have cancer treatment in the early disease status. As a result, early diagnosis plays an important role in cancer treatment. In chapter 2., we applied a validated GC-MS method to quantify fatty acids in plasma samples from both healthy volunteers and BC patients. Several fatty acids displayed significant difference between healthy control and BC group in both pre-menopausal and post-menopausal groups. These fatty acids provide the potential to be used as the BC detection biomarkers. Due to the small sample numbers, further verification with larger sample size is required to make these potential markers for clinical use. In addition, other parameters including BC molecular subtype and breastfeeding experience could be added in the prediction model to improve the prediction accuracy.

Therapeutic monoclonal antibody (mAb) based therapies were becoming more

doi:10.6342/NTU201804105

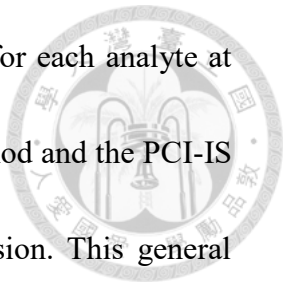
and more frequently applied to cancer treatment due to its' high efficacy and specificity, and also the low toxicity. These bio-pharmaceutical products have different pharmacokinetic (PK) and pharmacodynamic (PD) properties compared to conventional small chemical molecules. Therefore, it is important to have effective analytical methods to quantify these mAb drugs concentrations in bio-fluids which were commonly used in cancer therapy. Conventional enzyme-linked immunosorbent assay has been used in several studies for quantifying mAb drugs in human plasma samples, but this method has several limitations such as the cross reactivity problem. Mass spectrometer (MS) provide the high specificity and selectivity, and we developed two LC-MS/MS based analytical methods to quantify several mAb drugs. (chapter 3.) The sample matrix complexity may lead to the lower sensitivity, as a results, an appropriate sample preparation method to remove the interferences is really an important step in developing a quantitative analytical method. To reduce sample complexity, we applied a protein G purification strategy to trap bevacizumab in the sample matrix and followed by trypsin digestion to obtain the surrogate peptide of bevacizumab. To improve the method precision and accuracy, in chapter 3.1, we applied another immunoglobulin G (IgG) based therapeutic mAb drug as our internal standard to cover the potential variation during the sample preparation procedure in LC-MS/MS analysis. The developed approach was successfully applied to quantify bevacizumab

doi:10.6342/NTU201804105

concentrations in patients' plasma samples. It is anticipated that the developed method could provide useful drug concentration information to physicians for searching the optimal dose during drug therapy.

One of the advantages for MS is its' powerful potential to provide a general method to quantify different target analytes, which has a great value in clinical filed to facilitate various clinical studies. Considering the growing attentions to mAb drugs, and the majority of these mAb drugs belong to IgG class, it is important to develop a general analytical method for quantifing these IgG based mAbs for PK/PD studies and dose adjustment. We developed a general method to quantify IgG based therapeutic mAb drugs in plasma samples using bevacizumab, pembrolizumab and nivolumab as three demonstration drugs. (chapter 3.2) Protein G purification was applied for universally trapping IgG based mAbs. A major concern of using LC-MS to measure the mAb concentration in biological fluids is that the complexity of sample matrix may induce serious matrix effect (ME) during LC-MS analysis. To avoid the matrix effect that lead to inaccurate quantification results, it is critical not only to perform sample clean up but also apply a ME correction method in LC-MS analysis. Although stable isotopes labeled internal standards have been widely used in MS based analytical methods, it is not practical to purchase each SIL-IS for every target analyte, in addition, many of them were often not commercially available. To solve this problem, we applied a post-column

doi:10.6342/NTU201804105



infused internal standard (PCI-IS) to monitor and correct the ME for each analyte at different retention times. Combining the protein G purification method and the PCI-IS correction method could improve the method accuracy and precision. This general method was successfully applied to quantify several clinical samples under different mAb drugs treatments. We anticipate that this general method could be applied to other IgG based mAb drugs, providing a convenient, accurate, and reliable method to achieve precision medicine in the future.

Conclusively, biomarker discovery can help to achieve early disease detection, and the measurement of drug concentration in bio-fluid can provide additional information to the physicians to optimize the drug dosage. The proposed fatty acid markers and mAb quantification methods have potential to facilitate precision medicine. We anticipate that these developed methods could be applied to clinical use in the future and benefit more patients.

doi:10.6342/NTU201804105

Chapter 5. References

1. Hjartaker, A.; Aagnes, B.; Robsahm, T. E.; Langseth, H.; Bray, F.; Larsen, I. K., Subsite-specific dietary risk factors for colorectal cancer: a review of cohort studies. *J Oncol* **2013,** *2013*, 703854.

2. Gagnon, J.; Levesque, E.; Borduas, F.; Chiquette, J.; Diorio, C.; Duchesne, N.; Dumais, M.; Eloy, L.; Foulkes, W.; Gervais, N.; Lalonde, L.; L'Esperance, B.; Meterissian, S.; Provencher, L.; Richard, J.; Savard, C.; Trop, I.; Wong, N.; Knoppers, B. M.; Simard, J.; Canc, C. A. C. B., Recommendations on breast cancer screening and prevention in the context of implementing risk stratification: impending changes to current policies. *Curr Oncol* **2016,** *23* (6), E615-E625.

3. Ma, X.; Yang, Y.; Li, H. L.; Zheng, W.; Gao, J.; Zhang, W.; Yang, G.; Shu, X. O.; Xiang, Y. B., Dietary trace element intake and liver cancer risk: Results from two population-based cohorts in China. *Int. J. Cancer* **2017,** *140* (5), 1050-1059.

4. de Rooij, M.; Hamoen, E. H. J.; Futterer, J. J.; Barentsz, J. O.; Rovers, M. M., Accuracy of Multiparametric MRI for Prostate Cancer Detection: A Meta-Analysis. *Am J Roentgenol* **2014,** *202* (2), 343-351.

5. Rastinehad, A. R.; Turkbey, B.; Salami, S. S.; Yaskiv, O.; George, A. K.; Fakhoury, M.; Beecher, K.; Vira, M. A.; Kavoussi, L. R.; Siegel, D. N.; Villani, R.; Ben-Levi, E., Improving Detection of Clinically Significant Prostate Cancer: Magnetic Resonance

doi:10.6342/NTU201804105

Imaging/Transrectal Ultrasound Fusion Guided Prostate Biopsy. *J Urology* **2014,** *191* (6), 1749-1754.

6. Ng, E. K. O.; Li, R. F. N.; Shin, V. Y.; Jin, H. C.; Leung, C. P. H.; Ma, E. S. K.; Pang, R.; Chua, D.; Chu, K. M.; Law, W. L.; Law, S. Y. K.; Poon, R. T. P.; Kwong, A., Circulating microRNAs as Specific Biomarkers for Breast Cancer Detection. *Plos One* **2013,** *8* (1).

7. Kuhl, C. K.; Schrading, S.; Strobel, K.; Schild, H. H.; Hilgers, R. D.; Bieling, H. B., Abbreviated Breast Magnetic Resonance Imaging (MRI): First Postcontrast Subtracted Images and Maximum-Intensity Projection-A Novel Approach to Breast Cancer Screening With MRI. *J Clin Oncol* **2014,** *32* (22), 2304-U176.

8. Wu, P. F.; Lin, C. H.; Kuo, C. H.; Chen, W. W.; Yeh, D. C.; Liao, H. W.; Huang, S. M.; Cheng, A. L.; Lu, Y. S., A pilot study of bevacizumab combined with etoposide and cisplatin in breast cancer patients with leptomeningeal carcinomatosis. *Bmc Cancer* **2015,** *15*.

9. van Meir, H.; Kenter, G. G.; Burggraaf, J.; Kroep, J. R.; Welters, M. J. P.; Melief, C. J. M.; van der Burg, S. H.; van Poelgeest, M. I. E., The Need for Improvement of the Treatment of Advanced and Metastatic Cervical Cancer, the Rationale for Combined Chemo-Immunotherapy. *Anti-Cancer Agent Me* **2014,** *14* (2), 190-203.

10. Nickoloff, J. A., Improving cancer therapy by combining cell biological, physical,

doi:10.6342/NTU201804105

and molecular targeting strategies. *Chinese J Cancer Res* **2013,** *25* (1), 7-9.

11. La Vecchia, C.; Rota, M.; Malvezzi, M.; Negri, E., Potential for improvement in cancer management: reducing mortality in the European Union. *Oncologist* **2015,** *20* (5), 495-8.

12. Siegel, R. L.; Miller, K. D.; Jemal, A., Cancer Statistics, 2015. *Ca-Cancer J Clin* **2015,** *65* (1), 5-29.

13. Hashim, D.; Boffetta, P.; La Vecchia, C.; Rota, M.; Bertuccio, P.; Malvezzi, M.; Negri, E., The global decrease in cancer mortality: trends and disparities. *Ann Oncol* **2016,** *27* (5), 926-933.

14. Miller, K. D.; Siegel, R. L.; Lin, C. C.; Mariotto, A. B.; Kramer, J. L.; Rowland, J. H.; Stein, K. D.; Alteri, R.; Jemal, A., Cancer treatment and survivorship statistics, 2016. *Ca-Cancer J Clin* **2016,** *66* (4), 271-289.

15. Wardle, J.; Robb, K.; Vernon, S.; Waller, J., Screening for Prevention and Early Diagnosis of Cancer. *Am Psychol* **2015,** *70* (2), 119-133.

16. Strimbu, K.; Tavel, J. A., What are biomarkers? *Curr Opin Hiv Aids* **2010,** *5* (6), 463-466.

17. Ziegler, A.; Koch, A.; Krockenberger, K.; Grosshennig, A., Personalized medicine using DNA biomarkers: a review. *Hum Genet* **2012,** *131* (10), 1627-38.

18. Vargas, A. J.; Harris, C. C., Biomarker development in the precision medicine era:

doi:10.6342/NTU201804105

lung cancer as a case study. *Nat Rev Cancer* **2016,** *16* (8), 525-37.

19. Gharwan, H.; Groninger, H., Kinase inhibitors and monoclonal antibodies in oncology: clinical implications. *Nat Rev Clin Oncol* **2016,** *13* (4), 209-227.

20. Morrissey, K. M.; Yuraszeck, T. M.; Li, C. C.; Zhang, Y.; Kasichayanula, S., Immunotherapy and Novel Combinations in Oncology: Current Landscape, Challenges, and Opportunities. *Cts-Clin Transl Sci* **2016,** *9* (2), 89-104.

21. Clegg, L. X.; Reichman, M. E.; Miller, B. A.; Hankey, B. F.; Singh, G. K.; Lin, Y. D.; Goodman, M. T.; Lynch, C. F.; Schwartz, S. M.; Chen, V. W.; Bernstein, L.; Gomez, S. L.; Graff, J. J.; Lin, C. C.; Johnson, N. J.; Edwards, B. K., Impact of socioeconomic status on cancer incidence and stage at diagnosis: selected findings from the surveillance, epidemiology, and end results: National Longitudinal Mortality Study. *Cancer Cause Control* **2009,** *20* (4), 417-435.

22. DeSantis, C.; Siegel, R.; Bandi, P.; Jemal, A., Breast cancer statistics, 2011. *Ca-Cancer J Clin* **2011,** *61* (6), 409-418.

23. Ferlay, J.; Soerjomataram, I.; Dikshit, R.; Eser, S.; Mathers, C.; Rebelo, M.; Parkin, D. M.; Forman, D.; Bray, F., Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* **2015,** *136* (5), E359-86.

24. Rossi, S.; Cinini, C.; Dipietro, C.; Lombardi, C. P.; Crucitti, A.; Bellantone, R.;

doi:10.6342/NTU201804105

Crucitti, F., Diagnostic Delay in Breast-Cancer - Correlation with Disease Stage and Prognosis. *Tumori* **1990,** *76* (6), 559-562.

25. Nover, A. B.; Jagtap, S.; Anjum, W.; Yegingil, H.; Shih, W. Y.; Shih, W. H.; Brooks, A. D., Modern breast cancer detection: a technological review. *Int J Biomed Imaging* **2009,** *2009*, 902326.

26. Iqbal, J.; Ginsburg, O.; Rochon, P. A.; Sun, P.; Narod, S. A., Differences in Breast Cancer Stage at Diagnosis and Cancer-Specific Survival by Race and Ethnicity in the United States. *Jama-J Am Med Assoc* **2015,** *313* (2), 165-173.

27. Robinson, E.; Mohilever, J.; Borovik, R., Factors Affecting Delay in Diagnosis of Breast-Cancer - Relationship of Delay to Stage of Disease. *Israel J Med Sci* **1986,** *22* (5), 333-338.

28. Berg, W. A., Current Status of Supplemental Screening in Dense Breasts. *J Clin Oncol* **2016,** *34* (16), 1840-+.

29. Gierach, G. L.; Ichikawa, L.; Kerlikowske, K.; Brinton, L. A.; Farhat, G. N.; Vacek, P. M.; Weaver, D. L.; Schairer, C.; Taplin, S. H.; Sherman, M. E., Relationship Between Mammographic Density and Breast Cancer Death in the Breast Cancer Surveillance Consortium. *J Natl Cancer I* **2012,** *104* (16), 1218-1227.

30. Brown, P. O.; Botstein, D., Exploring the new world of the genome with DNA microarrays. *Nat Genet* **1999,** *21*, 33-37.

doi:10.6342/NTU201804105

31. Eckhart, A. D.; Beebe, K.; Milburn, M., Metabolomics as a Key Integrator for "Omic" Advancement of Personalized Medicine and Future Therapies. *Cts-Clin Transl Sci* **2012,** *5* (3), 285-288.

32. Wulfkuhle, J.; Espina, V.; Liotta, L.; Petricoin, E., Genomic and proteomic technologies for individualisation and improvement of cancer treatment. *Eur J Cancer* **2004,** *40* (17), 2623-2632.

33. Nicholson, J. K.; Lindon, J. C.; Holmes, E., 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica* **1999,** *29* (11), 1181-1189.

34. Woo, H. M.; Kim, K. M.; Choi, M. H.; Jung, B. H.; Lee, J.; Kong, G.; Nam, S. J.; Kim, S.; Bai, S. W.; Chung, B. C., Mass spectrometry based metabolomic approaches in urinary biomarker study of women's cancers. *Clin Chim Acta* **2009,** *400* (1-2), 63-69.

35. Putluri, N.; Shojaie, A.; Vasu, V. T.; Vareed, S. K.; Nalluri, S.; Putluri, V.; Thangjam, G. S.; Panzitt, K.; Tallman, C. T.; Butler, C.; Sana, T. R.; Fischer, S. M.; Sica, G.; Brat, D. J.; Shi, H. D.; Palapattu, G. S.; Lotan, Y.; Weizer, A. Z.; Terris, M. K.; Shariat, S. F.; Michailidis, G.; Sreekumar, A., Metabolomic Profiling Reveals Potential Markers and Bioprocesses Altered in Bladder Cancer Progression. *Cancer Res* **2011,** *71* (24), 7376-7386.

doi:10.6342/NTU201804105

36. Ganti, S.; Weiss, R. H., Urine metabolomics for kidney cancer detection and biomarker discovery. *Urol Oncol-Semin Ori* **2011,** *29* (5), 551-557.

37. Gowda, G. A. N., Human bile as a rich source of biomarkers for hepatopancreatobiliary cancers. *Biomark Med* **2010,** *4* (2), 299-314.

38. Cho, S. H.; Jung, B. H.; Lee, S. H.; Lee, W. Y.; Kong, G.; Chung, B. C., Direct determination of nucleosides in the urine of patients with breast cancer using column-switching liquid chromatography-tandem mass spectrometry. *Biomed Chromatogr* **2006,** *20* (11), 1229-1236.

39. Seidel, A.; Brunner, S.; Seidel, P.; Fritz, G. I.; Herbarth, O., Modified nucleosides: an accurate tumour marker for clinical diagnosis of cancer, early detection and therapy control. *Brit J Cancer* **2006,** *94* (11), 1726-1733.

40. Matsuda, F., Technical Challenges in Mass Spectrometry-Based Metabolomics. *Mass Spectrom (Tokyo)* **2016,** *5* (2), S0052.

41. Christians, U.; Klawitter, J.; Hornberger, A.; Klawitter, J., How Unbiased is Non-Targeted Metabolomics and is Targeted Pathway Screening the Solution? *Curr Pharm Biotechno* **2011,** *12* (7), 1053-1066.

42. Evans, A. M.; DeHaven, C. D.; Barrett, T.; Mitchell, M.; Milgram, E., Integrated, nontargeted ultrahigh performance liquid chromatography/electrospray ionization tandem mass spectrometry platform for the identification and relative quantification of

doi:10.6342/NTU201804105

the small-molecule complement of biological systems. *Anal Chem* **2009,** *81* (16), 6656-67.

43. Emwas, A. H.; Roy, R.; McKay, R. T.; Ryan, D.; Brennan, L.; Tenori, L.; Luchinat, C.; Gao, X.; Zeri, A. C.; Gowda, G. A. N.; Raftery, D.; Steinbeck, C.; Salek, R. M.; Wishart, D. S., Recommendations and Standardization of Biomarker Quantification Using NMR-Based Metabolomics with Particular Focus on Urinary Analysis. *J Proteome Res* **2016,** *15* (2), 360-373.

44. Scott, A. M.; Allison, J. P.; Wolchok, J. D., Monoclonal antibodies in cancer therapy. *Cancer Immun* **2012,** *12*, 14.

45. Ranpura, V.; Hapani, S.; Wu, S., Treatment-related mortality with bevacizumab in cancer patients: a meta-analysis. *JAMA* **2011,** *305* (5), 487-94.

46. Zhao, L.; Ren, T. H.; Wang, D. D., Clinical pharmacology considerations in biologics development. *Acta Pharmacol Sin* **2012,** *33* (11), 1339-47.

47. Glassman, P. M.; Balthasar, J. P., Mechanistic considerations for the use of monoclonal antibodies for cancer therapy. *Cancer Biol Med* **2014,** *11* (1), 20-33.

48. Yi, L. Z.; He, J.; Liang, Y. Z.; Yuan, D. L.; Chau, F. T., Plasma fatty acid metabolic profiling and biomarkers of type 2 diabetes mellitus based on GC/MS and PLS-LDA. *FEBS Lett* **2006,** *580* (30), 6837-45.

49. Nezami Ranjbar, M. R.; Luo, Y.; Di Poto, C.; Varghese, R. S.; Ferrarini, A.; Zhang,

doi:10.6342/NTU201804105

C.; Sarhan, N. I.; Soliman, H.; Tadesse, M. G.; Ziada, D. H.; Roy, R.; Ressom, H. W., GC-MS Based Plasma Metabolomics for Identification of Candidate Biomarkers for Hepatocellular Carcinoma in Egyptian Cohort. *Plos One* **2015,** *10* (6), e0127299.

50. Xie, G.; Lu, L.; Qiu, Y.; Ni, Q.; Zhang, W.; Gao, Y. T.; Risch, H. A.; Yu, H.; Jia, W., Plasma metabolite biomarkers for the detection of pancreatic cancer. *J Proteome Res* **2015,** *14* (2), 1195-202.

51. Tchu, S. M.; Lynch, K. L.; Wu, A. H., Therapeutic drug monitoring of tamoxifen using LC-MS/MS. *Methods Mol Biol* **2012,** *902*, 211-22.

52. Jacob, S.; Nair, A. B., An Updated Overview on Therapeutic Drug Monitoring of Recent Antiepileptic Drugs. *Drugs R D* **2016,** *16* (4), 303-316.

53. Yilmaz, B.; Sahin, H.; Erdem, A. F., Determination of naproxen in human plasma by GC-MS. *J Sep Sci* **2014,** *37* (8), 997-1003.

54. Annesley, T. M.; McKeown, D. A.; Holt, D. W.; Mussell, C.; Champarnaud, E.; Harter, L.; Calton, L. J.; Mason, D. S., Standardization of LC-MS for therapeutic drug monitoring of tacrolimus. *Clin Chem* **2013,** *59* (11), 1630-7.

55. Horning, E. C.; Horning, M. G., Metabolic profiles: gas-phase methods for analysis of metabolites. *Clin Chem* **1971,** *17* (8), 802-9.

56. Thompson, J. A.; Markey, S. P., Quantitative metabolic profiling of urinary organic acids by gas chromatography-mass spectrometry: comparison of isolation methods.

doi:10.6342/NTU201804105

Anal Chem **1975,** *47* (8), 1313-21.

57. Savolainen, O. I.; Sandberg, A. S.; Ross, A. B., A Simultaneous Metabolic Profiling and Quantitative Multimetabolite Metabolomic Method for Human Plasma Using Gas-Chromatography Tandem Mass Spectrometry. *J Proteome Res* **2016,** *15* (1), 259-65.

58. Zheng, X.; Qiu, Y.; Zhong, W.; Baxter, S.; Su, M.; Li, Q.; Xie, G.; Ore, B. M.; Qiao, S.; Spencer, M. D.; Zeisel, S. H.; Zhou, Z.; Zhao, A.; Jia, W., A targeted metabolomic protocol for short-chain fatty acids and branched-chain amino acids. *Metabolomics* **2013,** *9* (4), 818-827.

59. Fernie, A. R.; Trethewey, R. N.; Krotzky, A. J.; Willmitzer, L., Innovation - Metabolite profiling: from diagnostics to systems biology. *Nat Rev Mol Cell Bio* **2004,** *5* (9), 763-769.

60. Ecker, D. M.; Jones, S. D.; Levine, H. L., The therapeutic monoclonal antibody market. *Mabs-Austin* **2015,** *7* (1), 9-14.

61. Choi, B. K.; Gusev, A. I.; Hercules, D. M., Postcolumn introduction of an internal standard for quantitative LC-MS analysis. *Anal Chem* **1999,** *71* (18), 4107-4110.

62. Bonfiglio, R.; King, R. C.; Olah, T. V.; Merkle, K., The effects of sample preparation methods on the variability of the electrospray ionization response for model drug compounds. *Rapid Commun Mass Sp* **1999,** *13* (12), 1175-1185.

doi:10.6342/NTU201804105

63. Gonzalez, O.; van Vliet, M.; Damen, C. W. N.; van der Kloet, F. M.; Vreeken, R. J.; Hankemeier, T., Matrix Effect Compensation in Small-Molecule Profiling for an LC-TOF Platform Using Multicomponent Postcolumn Infusion. *Anal Chem* **2015,** *87* (12), 5921-5929.

64. Liao, H. W.; Chen, G. Y.; Tsai, I. L.; Kuo, C. H., Using a postcolumn-infused internal standard for correcting the matrix effects of urine specimens in liquid chromatography-electrospray ionization mass spectrometry. *J Chromatogr A* **2014,** *1327*, 97-104.

65. Liao, H. W.; Tsai, I. L.; Chen, G. Y.; Lu, Y. S.; Lin, C. H.; Kuo, C. H., Quantification of target analytes in various biofluids using a postcolumn infused-internal standard method combined with matrix normalization factors in liquid chromatography-electrospray ionization mass spectrometry. *J Chromatogr A* **2014,** *1358*, 85-92.

66. Budczies, J.; Denkert, C.; Muller, B. M.; Brockmoller, S. F.; Klauschen, F.; Gyorffy, B.; Dietel, M.; Richter-Ehrenstein, C.; Marten, U.; Salek, R. M.; Griffin, J. L.; Hilvo, M.; Oresic, M.; Wohlgemuth, G.; Fiehn, O., Remodeling of central metabolism in invasive breast cancer compared to normal breast tissue - a GC-TOFMS based metabolomics study. *Bmc Genomics* **2012,** *13*.

67. Lv, W.; Yang, T., Identification of possible biomarkers for breast cancer from free

doi:10.6342/NTU201804105

fatty acid profiles determined by GC-MS and multivariate statistical analysis. *Clinical Biochemistry* **2012,** *45* (1-2), 127-133.

68. Huang, C. H., Mixed-chain phospholipids: Structures and chain-melting behavior. *Lipids* **2001,** *36* (10), 1077-1097.

69. Buczynski, M. W.; Dumlao, D. S.; Dennis, E. A., An integrated omics analysis of eicosanoid biology. *J Lipid Res* **2009,** *50* (6), 1015-1038.

70. Calder, P. C., Polyunsaturated fatty acids, inflammation, and immunity. *Lipids* **2001,** *36* (9), 1007-24.

71. Leonelli, M.; Graciano, M. F.; Britto, L. R., TRP channels, omega-3 fatty acids, and oxidative stress in neurodegeneration: from the cell membrane to intracellular cross-links. *Braz. J. Med. Biol. Res.* **2011,** *44* (11), 1088-96.

72. Chua, M. E.; Sio, M. C.; Sorongon, M. C.; Dy, J. S., Relationship of dietary intake of omega-3 and omega-6 Fatty acids with risk of prostate cancer development: a meta-analysis of prospective studies and review of literature. *Prostate cancer* **2012,** *2012*, 826254.

73. MacLennan, M.; Ma, D. W., Role of dietary fatty acids in mammary gland development and breast cancer. *Breast Cancer Res.* **2010,** *12* (5), 211.

74. Lagerstedt, S. A.; Hinrichs, D. R.; Batt, S. M.; Magera, M. J.; Rinaldo, P.; McConnell, J. P., Quantitative determination of plasma c8-c26 total fatty acids for the

doi:10.6342/NTU201804105

biochemical diagnosis of nutritional and metabolic disorders. *Mol. Genet. Metab.* **2001,** *73* (1), 38-45.

75. Lamari, F.; Mochel, F.; Sedel, F.; Saudubray, J. M., Disorders of phospholipids, sphingolipids and fatty acids biosynthesis: toward a new category of inherited metabolic diseases. *J. Inherit. Metab. Dis.* **2013,** *36* (3), 411-25.

76. Calder, P. C., n-3 Fatty acids and cardiovascular disease: evidence explained and mechanisms explored. *Clin. Sci.* **2004,** *107* (1), 1-11.

77. Fiehn, O., Metabolomics - the link between genotypes and phenotypes. *Plant Mol Biol* **2002,** *48* (1-2), 155-171.

78. Sumner, L. W.; Mendes, P.; Dixon, R. A., Plant metabolomics: large-scale phytochemistry in the functional genomics era. *Phytochemistry* **2003,** *62* (6), 817-836.

79. Kell, D. B., Metabolomics and systems biology: making sense of the soup. *Curr Opin Microbiol* **2004,** *7* (3), 296-307.

80. Zhang, Y.; Song, L.; Liu, N.; He, C.; Li, Z., Decreased serum levels of free fatty acids are associated with breast cancer. *Clin Chim Acta* **2014,** *437*, 31-37.

81. Bannon, C. D.; Breen, G. J.; Craske, J. D.; Hai, N. T.; Harper, N. L.; Orourke, K. L., Analysis of fatty-acid methyl-esters with high-accuracy and reliability .3. Literature-review of and investigations into the development of rapid procedures for the methoxide-catalyzed methanolysis of fats and oils. *J Chromatogr* **1982,** *247* (1), 71-89.

doi:10.6342/NTU201804105

82. Aldai, N.; Murray, B. E.; Najera, A. I.; Troy, D. J.; Osoro, K., Derivatization of fatty acids and its application for conjugated linoleic acid studies in ruminant meat lipids. *J Sci Food Agric* **2005,** *85* (7), 1073-1083.

83. Masood, A.; Stark, K. D.; Salem, N., A simplified and efficient method for the analysis of fatty acid methyl esters suitable for large clinical studies. *J Lipid Res* **2005,** *46* (10), 2299-2305.

84. Delmonte, P.; Kia, A.-R. F.; Kramer, J. K. G.; Mossoba, M. M.; Sidisky, L.; Rader, J. I., Separation characteristics of fatty acid methyl esters using SLB-IL111, a new ionic liquid coated capillary gas chromatographic column. *J Chromatogr A* **2011,** *1218* (3), 545-554.

85. Ando, Y.; Sasaki, T., GC separation of cis-eicosenoic acid positional isomers on an ionic liquid SLB-IL100 stationary phase. *J Am Oil Chem Soc* **2011,** *88* (6), 743-748.

86. Delmonte, P.; Fardin-Kia, A. R.; Kramer, J. K. G.; Mossoba, M. M.; Sidisky, L.; Tyburczy, C.; Rader, J. I., Evaluation of highly polar ionic liquid gas chromatographic column for the determination of the fatty acids in milk fat. *J Chromatogr A* **2012,** *1233*, 137-146.

87. Gu, Q.; David, F.; Lynen, F.; Vanormelingen, P.; Vyverman, W.; Rumpel, K.; Xu, G.; Sandra, P., Evaluation of ionic liquid stationary phases for one dimensional gas chromatography-mass spectrometry and comprehensive two dimensional gas

doi:10.6342/NTU201804105

chromatographic analyses of fatty acids in marine biota. *J Chromatogr A* **2011,** *1218* (20), 3056-3063.

88. Ecker, J.; Scherer, M.; Schmitz, G.; Liebisch, G., A rapid GC-MS method for quantification of positional and geometric isomers of fatty acid methyl esters. *J Chromatogr B* **2012,** *897*, 98-104.

89. DeSantis, C.; Ma, J.; Bryan, L.; Jemal, A., Breast Cancer Statistics, 2013. *CA Cancer J Clin* **2014,** *64* (1), 52-62.

90. Lin, C. H.; Liau, J. Y.; Lu, Y. S.; Huang, C. S.; Lee, W. C.; Kuo, K. T.; Shen, Y. C.; Kuo, S. H.; Lan, C.; Liu, J. M.; Kuo, W. H.; Chang, K. J.; Cheng, A. L., Molecular subtypes of breast cancer emerging in young women in Taiwan: evidence for more than just westernization as a reason for the disease in Asia. *Cancer Epidem Biomar* **2009,** *18* (6), 1807-1814.

91. Walsh, G., Biopharmaceutical benchmarks 2014. *Nat Biotechnol* **2014,** *32* (10), 992-1000.

92. Hurwitz, H.; Fehrenbacher, L.; Novotny, W.; Cartwright, T.; Hainsworth, J.; Heim, W.; Berlin, J.; Baron, A.; Griffing, S.; Holmgren, E.; Ferrara, N.; Fyfe, G.; Rogers, B.; Ross, R.; Kabbinavar, F., Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *New Engl J Med* **2004,** *350* (23), 2335-2342.

93. Mulleman, D.; Meric, J. C.; Paintaud, G.; Ducourau, E.; Magdelaine-Beuzelin, C.;

doi:10.6342/NTU201804105

Valat, J. P.; Goupille, P.; GICC, C. N. R. S. U., Infliximab concentration monitoring improves the control of disease activity in rheumatoid arthritis. *Arthritis Res Ther* **2009,** *11* (6).

94. Wang, W.; Wang, E. Q.; Balthasar, J. P., Monoclonal Antibody Pharmacokinetics and Pharmacodynamics. *Clin Pharmacol Ther* **2008,** *84* (5), 548-558.

95. Ranpura, V.; Hapani, S.; Wu, S. H., Treatment-Related Mortality With Bevacizumab in Cancer Patients A Meta-analysis. *Jama-J Am Med Assoc* **2011,** *305* (5), 487-494.

96. Damen, C. W. N.; de Groot, E. R.; Heij, M.; Boss, D. S.; Schellens, J. H. M.; Rosing, H.; Beijnen, J. H.; Aarden, L. A., Development and validation of an enzyme-linked immunosorbent assay for the quantification of trastuzumab in human serum and plasma. *Anal Biochem* **2009,** *391* (2), 114-120.

97. Ternant, D.; Mulleman, D.; Degenne, D.; Willot, S.; Guillaumin, J. M.; Watier, H.; Goupille, P.; Paintaud, G., An enzyme-linked immunosorbent assay for therapeutic drug monitoring of infliximab. *Ther Drug Monit* **2006,** *28* (2), 169-174.

98. Kim, W.; Lee, J. E.; Li, X. F.; Kim, S. H.; Han, B. G.; Lee, B. I.; Kim, J. K.; Choi, K.; Kim, H. J., Quantitative measurement of anti-aquaporin-4 antibodies by enzyme-linked immunosorbent assay using purified recombinant human aquaporin-4. *Mult Scler J* **2012,** *18* (5), 578-586.

doi:10.6342/NTU201804105

99. O'Hara, D. M.; Theobald, V.; Egan, A. C.; Usansky, J.; Krishna, M.; TerWee, J.; Maia, M.; Spriggs, F. P.; Kenney, J.; Safavi, A.; Keefe, J., Ligand Binding Assays in the 21st Century Laboratory: Recommendations for Characterization and Supply of Critical Reagents. *Aaps J* **2012,** *14* (2), 316-328.

100. Staack, R. F.; Stracke, J. O.; Stubenrauch, K.; Vogel, R.; Schleypen, J.; Papadimitriou, A., Quality requirements for critical assay reagents used in bioanalysis of therapeutic proteins: what bioanalysts should know about their reagents. *Bioanalysis* **2011,** *3* (5), 523-534.

101. Savoie, N.; Garofolo, F.; van Amsterdam, P.; Bansal, S.; Beaver, C.; Bedford, P.; Booth, B. P.; Evans, C.; Jemal, M.; Lefebvre, M.; de Silva, A. L. L.; Lowes, S.; Marini, J. C.; Masse, R.; Mawer, L.; Ormsby, E.; Rocci, M. L.; Viswanathan, C. T.; Wakelin-Smith, J.; Welink, J.; White, J. T.; Woolf, E., 2010 White Paper on Recent Issues in Regulated Bioanalysis & Global Harmonization of Bioanalytical Guidance. *Bioanalysis* **2010,** *2* (12), 1945-1960.

102. Casteele, N. V.; Buurman, D. J.; Sturkenboom, M. G. G.; Kleibeuker, J. H.; Vermeire, S.; Rispens, T.; van der Kleij, D.; Gils, A.; Dijkstra, G., Detection of infliximab levels and anti-infliximab antibodies: a comparison of three different assays. *Aliment Pharm Ther* **2012,** *36* (8), 765-771.

103. Yang, Z. P.; Hayes, M.; Fang, X. P.; Daley, M. P.; Ettenberg, S.; Tse, F. L. S., LC-

doi:10.6342/NTU201804105

MS/MS approach therapeutic proteins for quantification of in plasma using a protein internal standard and 2D-solid-phase extraction cleanup. *Anal Chem* **2007,** *79* (24), 9294-9301.

104. Jourdil, J. F.; Lebert, D.; Gautier-Veyret, E.; Lemaitre, F.; Bonaz, B.; Picard, G.; Tonini, J.; Stanke-Labesque, F., Infliximab quantitation in human plasma by liquid chromatography-tandem mass spectrometry: towards a standardization of the methods? *Anal Bioanal Chem* **2017,** *409* (5), 1195-1205.

105. Bults, P.; van de Merbel, N. C.; Bischoff, R., Quantification of biopharmaceuticals and biomarkers in complex biological matrices: a comparison of liquid chromatography coupled to tandem mass spectrometry and ligand binding assays. *Expert Rev Proteomic* **2015,** *12* (4), 355-374.

106. Hopfgartner, G.; Lesur, A.; Varesio, E., Analysis of biopharmaceutical proteins in biological matrices by LC-MS/MS II. LC-MS/MS analysis. *Trac-Trend Anal Chem* **2013,** *48*, 52-61.

107.Stojiljkovic, N.; Paris, A.; Garcia, P.; Popot, M. A.; Bonnaire, Y.; Tabet, J. C.; Junot, C., Application and challenges in using LC-MS assays for absolute quantitative analysis of therapeutic proteins in drug discovery. *Bioanalysis* **2014,** *6* (6), 785-803.

108. Iwamoto, N.; Umino, Y.; Aoki, C.; Yamane, N.; Hamada, A.; Shimada, T., Fully validated LCMS bioanalysis of Bevacizumab in human plasma using nano-surface and

doi:10.6342/NTU201804105

molecular-orientation limited (nSMOL) proteolysis. *Drug Metab. Pharmacokinet.* **2016,** *31* (1), 46-50.

109. Lu, Q. Z.; Zheng, X. Y.; McIntosh, T.; Davis, H.; Nemeth, J. F.; Pendley, C.; Wu, S. L.; Hancock, W. S., Development of Different Analysis Platforms with LC-MS for Pharmacokinetic Studies of Protein Drugs. *Anal Chem* **2009,** *81* (21), 8715-8723.

110. Ocana, M. F.; James, I. T.; Kabir, M.; Grace, C.; Yuan, G. J.; Martin, S. W.; Neubert, H., Clinical Pharmacokinetic Assessment of an Anti-MAdCAM Monoclonal Antibody Therapeutic by LC-MS/MS. *Anal Chem* **2012,** *84* (14), 5959-5967.

111. Li, H. Y.; Ortiz, R.; Tran, L.; Hall, M.; Spahr, C.; Walker, K.; Laudernann, J.; Miller, S.; Salimi-Moosavi, H.; Lee, J. W., General LC-MS/MS Method Approach to Quantify Therapeutic Monoclonal Antibodies Using a Common Whole Antibody Internal Standard with Application to Preclinical Studies. *Anal Chem* **2012,** *84* (3), 1267-1273.

112. Todoroki, K.; Nakano, T.; Eda, Y.; Ohyama, K.; Hayashi, H.; Tsuji, D.; Min, J. Z.; Inoue, K.; Iwamoto, N.; Kawakami, A.; Ueki, Y.; Itoh, K.; Toyo'oka, T., Bioanalysis of bevacizumab and infliximab by high-temperature reversed-phase liquid chromatography with fluorescence detection after immunoaffinity magnetic purification. *Anal Chim Acta* **2016,** *916*, 112-119.

113. Iwamoto, N.; Shimada, T.; Umino, Y.; Aoki, C.; Aoki, Y.; Sato, T. A.; Hamada, A.; Nakagama, H., Selective detection of complementarity-determining regions of

doi:10.6342/NTU201804105

monoclonal antibody by limiting protease access to the substrate: nano-surface and molecular-orientation limited proteolysis. *Analyst* **2014,** *139* (3), 576-80.

114. Gonzalez-Quintela, A.; Alende, R.; Gude, F.; Campos, J.; Rey, J.; Meijide, L. M.; Fernandez-Merino, C.; Vidal, C., Serum levels of immunoglobulins (IgG, IgA, IgM) in a general adult population and their relationship with alcohol consumption, smoking and common metabolic abnormalities. *Clin Exp Immunol* **2008,** *151* (1), 42-50.

115. Peng, X. Y.; Liu, B. N.; Li, Y. T.; Wang, H.; Chen, X.; Guo, H. Z.; Guo, Q. C.; Xu, J.; Wang, H.; Zhang, D. P.; Dai, J. X.; Hou, S.; Guo, Y. J., Development and Validation of LC-MS/MS Method for the Quantitation of Infliximab in Human Serum. *Chromatographia* **2015,** *78* (7-8), 521-531.

116. Dispenzieri, A.; Gertz, M. A.; Therneau, T. M.; Kyle, R. A., Retrospective cohort study of 148 patients with polyclonal gammopathy. *Mayo Clin Proc* **2001,** *76* (5), 476-487.

117. DeSilva, B.; Smith, W.; Weiner, R.; Kelley, M.; Smolec, J. M.; Lee, B.; Khan, M.; Tacey, R.; Hill, H.; Celniker, A., Recommendations for the bioanalytical method validation of ligand-binding assays to support pharmacokinetic assessments of macromolecules. *Pharm Res* **2003,** *20* (11), 1885-1900.

118. El Amrani, M.; van den Broek, M. P. H.; Gobel, C.; van Maarseveen, E. M., Quantification of active infliximab in human serum with liquid chromatography-

doi:10.6342/NTU201804105

tandem mass spectrometry using a tumor necrosis factor alpha-based pre-analytical sample purification and a stable isotopic labeled infliximab bio-similar as internal standard: A target-based, sensitive and cost-effective method. *J Chromatogr A* **2016,** *1454*, 42-48.

119. Ferrara, N.; Hillan, K. J.; Gerber, H. P.; Novotny, W., Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. *Nat Rev Drug Discov* **2004,** *3* (5), 391-400.

120. Lanshoeft, C.; Wolf, T.; Heudi, O.; Cianferani, S.; Barteau, S.; Walles, M.; Picard, F.; Kretz, O., The use of generic surrogate peptides for the quantitative analysis of human immunoglobulin G1 in pre-clinical species with high-resolution mass spectrometry. *Anal Bioanal Chem* **2016,** *408* (6), 1687-1699.

121. Bender, J. L. G.; Adamson, P. C.; Reid, J. M.; Xu, L.; Baruchel, S.; Shaked, Y.; Kerbel, R. S.; Cooney-Qualter, E. M.; Stempak, D.; Chen, H. X.; Nelson, M. D.; Krailo, M. D.; Ingle, A. M.; Blaney, S. M.; Kandel, J. J.; Yamashiro, D. J., Phase I trial and pharmacokinetic study of bevacizumab in pediatric patients with refractory solid tumors: A children's oncology group study. *J Clin Oncol* **2008,** *26* (3), 399-405.

122. Lu, J. F.; Bruno, R.; Eppler, S.; Novotny, W.; Lum, B.; Gaudreault, J., Clinical pharmacokinetics of bevacizumab in patients with solid tumors. *Cancer Chemoth Pharm* **2008,** *62* (5), 779-786.

doi:10.6342/NTU201804105

123. Ternant, D.; Ceze, N.; Lecomte, T.; Degenne, D.; Duveau, A. C.; Watier, H.; Dorval, E.; Paintaud, G., An Enzyme-Linked Immunosorbent Assay to Study Bevacizumab Pharmacokinetics. *Ther Drug Monit* **2010,** *32* (5), 647-652.

124. Nugue, G.; Bidart, M.; Arlotto, M.; Mousseau, M.; Berger, F.; Pelletier, L., Monitoring Monoclonal Antibody Delivery in Oncology: The Example of Bevacizumab. *Plos One* **2013,** *8* (8).

125. Reichert, J. M., Antibodies to watch in 2014. *Mabs-Austin* **2014,** *6* (1), 5-14.

126.Borghaei, H.; Paz-Ares, L.; Horn, L.; Spigel, D. R.; Steins, M.; Ready, N. E.; Chow, L. Q.; Vokes, E. E.; Felip, E.; Holgado, E.; Barlesi, F.; Kohlhaufl, M.; Arrieta, O.; Burgio, M. A.; Fayette, J.; Lena, H.; Poddubskaya, E.; Gerber, D. E.; Gettinger, S. N.; Rudin, C. M.; Rizvi, N.; Crino, L.; Blumenschein, G. R.; Antonia, S. J.; Dorange, C.; Harbison, C. T.; Finckenstein, F. G.; Brahmer, J. R., Nivolumab versus Docetaxel in Advanced Nonsquamous Non-Small-Cell Lung Cancer. *New Engl J Med* **2015,** *373* (17), 1627-1639.

127. Chan, A. C.; Carter, P. J., Therapeutic antibodies for autoimmunity and inflammation. *Nat Rev Immunol* **2010,** *10* (5), 301-316.

128. Hortobagyi, G. N., Trastuzumab in the treatment of breast cancer. *New Engl J Med* **2005,** *353* (16), 1734-1736.

129. McCormack, P. L.; Keam, S. J., Bevacizumab - A review of its use in metastatic

doi:10.6342/NTU201804105

colorectal cancer. *Drugs* **2008,** *68* (4), 487-506.

130. Munnink, T. H. O.; Henstra, M. J.; Segerink, L. I.; Movig, K. L. L.; Brummelhuis-Visser, P., Therapeutic Drug Monitoring of Monoclonal Antibodies in Inflammatory and Malignant Disease: Translating TNF-alpha Experience to Oncology. *Clin Pharmacol Ther* **2016,** *99* (4), 419-431.

131. Vozy, A.; Zalcman, G., Pembrolizumab for the Treatment of Non-Small-Cell Lung Cancer. *Oncologie* **2015,** *17* (9), 407-408.

132. Rugo, H. S.; Delord, J. P.; Im, S. A.; Ott, P. A.; Piha-Paul, S. A.; Bedard, P. L.; Sachdev, J.; Le Tourneau, C.; van Brummelen, E.; Varga, A.; Saraf, S.; Pietrangelo, D.; Karantza, V.; Tan, A., Preliminary efficacy and safety of pembrolizumab (MK-3475) in patients with PD-L1 positive, estrogen receptor-positive (ER+)/HER2-negative advanced breast cancer enrolled in KEYNOTE-028. *Cancer Res.* **2016,** *76*.

133. Gao, B.; Yeap, S.; Clements, A.; Balakrishnar, B.; Wong, M.; Gurney, H., Evidence for Therapeutic Drug Monitoring of Targeted Anticancer Therapies. *J Clin Oncol* **2012,** *30* (32), 4017-4025.

134. Minasian, L.; Rosen, O.; Auclair, D.; Rahman, A.; Pazdur, R.; Schilsky, R. L., Optimizing Dosing of Oncology Drugs. *Clin Pharmacol Ther* **2014,** *96* (5), 572-579.

135. Xu, K. Y.; Liu, L. N.; Maia, M.; Li, J.; Lowe, J.; Song, A.; Kaur, S., A multiplexed hybrid LC-MS/MS pharmacokinetic assay to measure two co-administered monoclonal

doi:10.6342/NTU201804105

antibodies in a clinical study. *Bioanalysis* **2014,** *6* (13), 1781-1794.

136. Iwamoto, N.; Yamane, N.; Umino, Y.; Hamada, A.; Shimada, T., The development of the validated LCMS bioanalysis of trastuzumab in human plasma using a selective detection method for complementarity-determining regions of monoclonal antibodies: nano-surface and molecular-orientation limited (nSMOL) proteolysis. *Anal Methods-Uk* **2015,** *7* (21), 9177-9183.

137. Iwamoto, N.; Shimada, T.; Terakado, H.; Hamada, A., Validated LC-MS/MS analysis of immune checkpoint inhibitor Nivolumab in human plasma using a Fab peptide-selective quantitation method: nano-surface and molecular-orientation limited (nSMOL) proteolysis. *J Chromatogr B* **2016,** *1023*, 9-16.

138. El Amrani, M.; van den Broek, M. P.; Gobel, C.; van Maarseveen, E. M., Quantification of active infliximab in human serum with liquid chromatography-tandem mass spectrometry using a tumor necrosis factor alpha -based pre-analytical sample purification and a stable isotopic labeled infliximab bio-similar as internal standard: A target-based, sensitive and cost-effective method. *J Chromatogr A* **2016,** *1454*, 42-8.

139. Iwamoto, N.; Shimada, T.; Terakado, H.; Hamada, A., Validated LC-MS/MS analysis of immune checkpoint inhibitor Nivolumab in human plasma using a Fab peptide-selective quantitation method: nano-surface and molecular-orientation limited

doi:10.6342/NTU201804105

(nSMOL) proteolysis. *J Chromatogr B* **2016,** *1023-1024*, 9-16.

140. Kazandjian, D.; Suzman, D. L.; Blumenthal, G.; Mushti, S.; He, K.; Libeg, M.; Keegan, P.; Pazdur, R., FDA Approval Summary: Nivolumab for the Treatment of Metastatic Non-Small Cell Lung Cancer With Progression On or After Platinum-Based Chemotherapy. *Oncologist* **2016,** *21* (5), 634-642.

141. Carbognin, L.; Pilotto, S.; Milella, M.; Vaccaro, V.; Brunelli, M.; Calio, A.; Cuppone, F.; Sperduti, I.; Giannarelli, D.; Chilosi, M.; Bronte, V.; Scarpa, A.; Bria, E.; Tortora, G., Differential Activity of Nivolumab, Pembrolizumab and MPDL3280A according to the Tumor Expression of Programmed Death-Ligand-1 (PD-L1): Sensitivity Analysis of Trials in Melanoma, Lung and Genitourinary Cancers. *Plos One* **2015,** *10* (6).

142. Ivashko, I. N.; Kolesar, J. M., Pembrolizumab and nivolumab: PD-1 inhibitors for advanced melanoma. *Am J Health-Syst Ph* **2016,** *73* (4), 193-201.

143. Team, R. D. C., *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing: 2008.

144. Chiu, H. H.; Tsai, I. L.; Lu, Y. S.; Lin, C. H.; Kuo, C. H., Development of an LC-MS/MS method with protein G purification strategy for quantifying bevacizumab in human plasma. *Anal Bioanal Chem* **2017,** 409(28), 6583-6593.

145. Mullard, A., 2016 FDA drug approvals. *Nat Rev Drug Discov* **2017,** *16* (2), 73-76.

doi:10.6342/NTU201804105

Publication list

1. **Chiu, H. H.**; Liao, H. W.; Shao, Y. Y.; Lu, Y. S.; Lin, C. H.; Tsai, I. L.; Kuo, C. H., Development of a general method for quantifying IgG-based therapeutic monoclonal antibodies in human plasma using protein G purification coupled with a two internal standard calibration strategy using LC-MS/MS. *Anal Chim Acta* **2018**, 1019, 93-102.

2. **Chiu, H. H.**; Tsai, I. L.; Lu, Y. S.; Lin, C. H.; Kuo, C. H., Development of an LC-MS/MS method with protein G purification strategy for quantifying bevacizumab in human plasma. *Anal Bioanal Chem* **2017**, 409 (28), 6583-6593.

3. **Chiu, H. H.**; Tsai, S. J.; Tseng, Y. J.; Wu, M. S.; Liao, W. C.; Huang, C. S.; Kuo, C. H., An efficient and robust fatty acid profiling method for plasma metabolomic studies by gas chromatography-mass spectrometry. *Clin Chim Acta* **2015**, 451, 183-190.

4. Chen, G. Y.; **Chiu, H. H.**; Lin, S. W.; Tseng, Y. J.; Tsai, S. J.; Kuo, C. H., Development and application of a comparative fatty acid analysis method to investigate voriconazole-induced hepatotoxicity. *Clin Chim Acta* **2015**, 438, 126-134.

doi:10.6342/NTU201804105