Development and validation of UPLC-MS/MS methods for simultaneous determination of (-)-epigallocatechin-3-gallate and decitabine in plasma

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Abstract

Epigallocatechin-3-gallate (EGCG) is an active substance extracted from green tea, and decitabine (DAC) is a DNA demethylating agent. It is found that the combination of these two compounds inhibits triple negative breast cancer tumor growth in a human xenograft mouse model by blocking the activation of Wnt signaling pathway. In order to quantitate compounds in biological samples for further pharmacokinetic studies, our objective was to establish a method for simultaneous determination of EGCG and DAC in plasma using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). The regression equations of EGCG and DAC detector response versus concentration were $y = 0.0051x - 2.4367$ ($r^2 = 0.98$) and $y = 0.00001x - 0.0013$ ($r^2 = 1.0$), respectively. The recovery rate, accuracy and precision of this method still need further validation.

Acknowledgements

I would like to thank Dr. Greenblatt, Dr. Yee and their lab members, especially Ms. Su Xiang Duan, who played an important role in my lab skill and academic training and supervising this thesis.

At the time I wrote this thesis, the entire world has been facing a large challenge from COVID-19. All campuses and laboratories were shut down. I would like to thank all faculties and staffs for their efforts to maintain school order and support students' life. I would also like to thank my family and my friends who always stand behind me and support me with love.

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List of Abbreviations

APC	Adenomatous polyposis coli
BEH	Ethylene bridged hybrid
CK1	Casein kinase 1
CV	Coefficient of variation
DAC	Decitabine
EGC	(-)-Epigallocatechin
EGCG	(-)-epigallocatechin-3-gallate
ER	Estrogen receptor
ESI	Electrospray ion source
FZD	Frizzled
GSK3β	Glycogen synthase kinase-3β
HER2	Human epidermal growth factor receptor 2
HPLC	High performance liquid chromatography
IS	Internal standard
LLOQ	Lower limit of quantification
LRP5/6	Low-density lipoprotein receptor-related protein 5 or 6
MRM	Multiple reaction monitoring

Chapter 1: Introduction

Triple-negative breast cancer (TNBC) lacks expression of estrogen receptor (ER-negative), progesterone receptor (PR-negative), and human epidermal growth factor receptor 2 (HER2-negative) [1]. TNBC accounts for about 9–21 % of all breast cancers, including patients with all the stages of breast cancer [2]. The maximum risk of recurrence of TNBC is between the first and third year following diagnosis, and survival after recurrence is significantly shorter than that observed in patients with non-triple-negative controls [3,4]. Also, brain metastases are the most common complication, with very poor prognosis [5]. TNBC does not respond to hormone treatment or anti-HER2 treatment, so chemotherapy is the main treatment for such patients [6]. However, intolerable side effects associated with chemotherapy and development of chemo-resistance limit the success of chemotherapy in TNBC [6]. Thus, finding novel therapeutic options to treat TNBC is of great interest.

Activation of the Wnt signaling is a common feature of TNBC [7]. In the absence of Wnt proteins, β-catenin is sequestered in a complex that consists of the adenomatous polyposis coli (APC) tumor suppressor, axin, glycogen synthase kinase-3β (GSK3β), and casein kinase 1 (CK1). This complex formation induces the phosphorylation of β-catenin by CK1 and GSK3β, which results in the subsequent degradation of β-catenin [8]. Conversely, when Wnt proteins are secreted from cells they can form a ternary complex with Frizzled (FZD) and the low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6) receptors, which results in the inhibition of GSK3β and the stabilization

of cytosolic β-catenin. The β-catenin then translocates into the nucleus where it interacts with T-cell factor/lymphoid enhancing factor to induce the expression of downstream target genes that regulate cell cycle, growth, and progression [8].

Previous research from Dr.Yee's laboratory has shown that a combination therapy of (-)-epigallocatechin-3-gallate (EGCG, figure 1.1A), an active ingredient extracted from green tea, and decitabine (DAC, figure 1.1B), a clinically available DNA demethylating agent, inhibits TNBC tumor growth in a human xenograft mouse model that is immune-compromised (lack of B-cells and T-cells) by blocking the activation of the Wnt signaling pathway. The combination drug can cross blood-brain-barrier and thus the therapy also decreases the incidence of brain metastases. Surprisingly, transcriptomic and bioinformatics analysis using RNAseq data from EGCG/DAC-treated tumor suggests a re-activation of the antigen-presenting pathways and upregulation of the genes expression involved in anti-tumor immune response and interferon signaling pathway in TNBC.

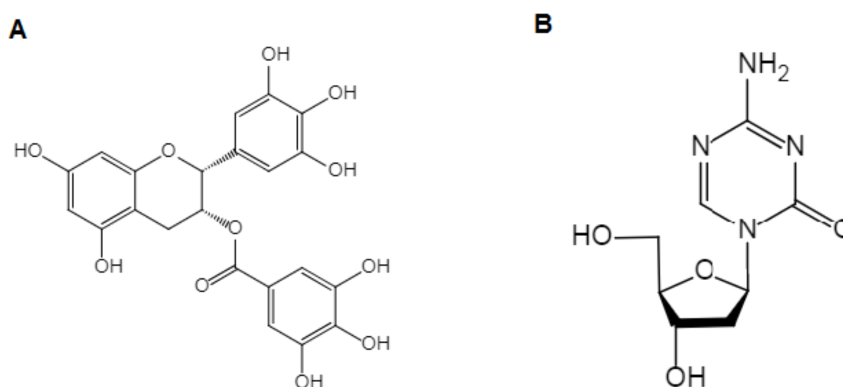


Figure 1.1 Chemical structure of (-)-epigallocatechin-3-gallate (A) and decitabine (B). The figure was drew using InDraw version 5.2 (Integle, Shanghai, China).

Since the combination of EGCG and DAC has already shown a strong preclinical results, this study will focus on development of a method for simultaneous determination of EGCG and DAC in plasma, for purpose of providing and provide an effective and reliable method for pharmacokinetic study.

Pharmacokinetics is a study about drugs or their metabolites absorption, distribution, metabolism and excretion in vivo. Pharmacokinetics plays an important role in studying drug's safety, effectiveness, and controllability. In order to obtain the best therapeutic effect and determine different dosing schemes for different patients, it is necessary to understand the changes in the absorption, distribution, metabolism and excretion of drugs in the body. Research on interaction of drug and body provides reference information for clinical trials and rational drug use. It is of great importance in the process of drug development and clinical use.

Pharmacokinetic parameters (such as rate constants for absorption, distribution and elimination) are used to describe the changes in drug concentrations and amounts throughout the body. The most commonly used method of pharmacokinetics is to determine drug concentration in vivo corresponding to multiple time points after administration and use software to calculate the pharmacokinetic parameters. At present, the methods used for in vivo drug analysis mainly include spectroscopy, chromatography, immunoassay and microbiology, especially ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS).

Low drug concentration and small amount of sample are characteristics of

biological sample analysis. UPLC-MS/MS is characterised as having high separation ability, strong selectivity, high sensitivity and the capacity to provide some analyte molecular weight and structural information. Compared to high performance liquid chromatography (HPLC), UPLC-MS/MS has high sensitivity, and through multiple response monitoring mode its signal-noise ratio can also be significantly improved thus reducing lower detection limit. These features make UPLC-MS/MS more suitable for biological analysis.

Song Y et al [9] developed a LC-MS/MS method for determination of EGCG in rat plasma and different tissues. Li J et al [10] developed a simultaneous determination method of A-type EGCG and ECG dimers in mouse plasma and its metabolites by UPLC-MS/MS. Zhang Y et al [11] and Xu H et al [12] developed methods for determination of DAC in rat plasma using HPLC-MS/MS. However, no study has been done developing a method for simultaneously determination of EGCG and DAC in plasma. This study provides a possible method for the combination of two drugs to be determined simultaneously by UPLC-MS/MS.

Chapter 2: Materials and Methods

2.1. Chemicals and reagents

The chromatographic standards for EGCG (≥95% purity), DAC (≥97% purity), diazepam, KH_2PO_4 and bovine serum (adult) were purchased from Sigma-Aldrich (St. Louis, MO, USA). (-)-Epigallocatechin (EGC) was purchased from Cayman Chemical (Ann Arbor, MI, USA). Methanol (LC-MS grade), 0.1% formic acid in acetonitrile (LC-MS grade), 0.1% formic acid in water (LC-MS grade), ethyl acetate (ACS grade) and 2-propanol (LC-MS grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA).

2.2. Instrumentation

The analyses were performed on an ultra-performance liquid chromatography system (ACQUITY UPLC, Waters Corp., Milford, MA, USA) coupled to a QTRAP 5500 mass spectrometer (Applied Biosystems/MDS SCIEX, Toronto, Canada) with an electrospray ion source (ESI). Analytes were separated by an Ethylene Bridged Hybrid (BEH) C_8 column (2.1 mm × 50 mm, 1.7 μm, Waters Corp., Wexford, Ireland). All data were acquired and processed by Analyst Software version 1.6.2 (Waters Corp., Milford, MA, USA).

2.3. UPLC-MS/MS conditions

2.3.1. Chromatography

EGC is another catechin that also found in the high amount in green tea. For analysis of EGCG, EGC was used as internal standard (IS). Gradient elution was

conducted with 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) as follows: 0-1.2 min (50% A), 1.2-3.6 min (40% A), 3.6-4 min (30% A), 4-5 min (50% A). For analysis of DAC, diazepam was used as IS. Gradient elution was conducted with 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) as follows: 0-4 min (90% A), 4-4.1 min (5% A), 4.1-5 min (90% A). For both analyses, the flow rate was 0.3 mL/min, column temperature was 40 ℃, sample temperature was 8 ℃ and injection volume was 2 μL. Injection wash solvents were methanol-water (5:95, v/v) and methanol-acetonitrile-2-propanol-water (1:1:1:1, v/v) for weak and strong wash, respectively.

2.3.2. Mass spectrometry

For EGCG analysis, data acquisition was performed under negative ESI mode. For DAC analysis, data acquisition was performed under positive ESI mode. Multiple reaction monitoring (MRM) mode was used for both analyses. General and specific analytes mass spectrometric parameters are listed in Table 2.1.

2.4. Preparation of standard solutions, calibration standards and quality control samples

Standard stock solution of EGCG, EGC, DAC and diazepam were prepared by dissolving the standard compounds in methanol individually, yielding concentrations of 5 mg/mL for EGCG, 0.1 mg/mL for EGC, 0.5 mg/mL for DAC and 0.1 mg/mL for diazepam. Working solutions for calibration curve and quality control (QC) samples were prepared by diluting stock solution with methanol. EGC and diazepam as IS were diluted to 10000 ng/mL and 1000ng/mL, respectively. All stock solutions were stored

at -20 ℃.

Table 2.1 General and specific mass spectrometric parameters.

General settings		
Analyte	EGCG	DAC
Curtain gas (psi)	15	15
Collision Gas	Medium	Medium
Ionspray voltage (V)	-4500	5000
Temperature (℃)	300	300
Ion source gas 1 (psi)	20	20
Ion source gas 2 (psi)	0	0

Specific settings				
Analyte	EGCG	EGC	DAC	Diazepam
Q1 Mass (Da)	457.000	305.100	229.981	285.000
Q3 Mass (Da)	168.900	124.800	113.000	193.000
Time (msec)	200	200	200	200
Declustering potential (V)	-74	-45	56	56
Entrance potential (V)	-10	-10	10	10
Collision energy (V)	-30	-33	19	33
Collision cell exit potential (V)	-1	-1	8	8

Samples for calibration curves were prepared by adding 10 μL of each analyte working solution and 10 μL of each IS working solution to blank plasma to obtain concentrations of 10-25000 ng/mL (EGCG) and 10-10000 ng/mL (DAC). The samples were mixed with 1mL of ethyl acetate for liquid-liquid extraction to extract analytes from plasma. After vortexing and centrifuging at 14000 rpm for 10 min, 800 μL of the upper organic layer was collected. The solvent was evaporated under vacuum and then redissolved in 250 μL acetonitrile: water (1:1, v/v). An aliquot of 200 μL was transferred to the autosampler vial for LC-MS/MS analysis.

Quality control samples (QCs) were prepared by spiking analyte working solution into plasma in the same way as the calibration samples. The plasma concentrations of

QCs were 10 and 10 (QC lower limit of quantification), 25 and 25 (QC low concentration), 12500 and 5000 (QC medium concentration), 20000 and 8000 (QC high concentration) ng/mL for EGCG and DAC, respectively.

Chapter 3: Results

3.1. Method development

In preliminary studies, assays of EGCG and DAC were assessed using Agilent HPLC with UV detection. The UV absorption spectrum of EGCG and DAC (Figure 3.1) suggests DAC has maximal UV absorption at 192 nm, and EGCG has an absorption at around 288 nm. Separation was performed using a Waters ACQUITY μBondapak C18 column (3.9 × 300 mm, Waters Corporation, Milford, MA, U.S.A.). The column temperature was maintained at 30 °C. The mobile phase consisted of 80% 20 Mm KH_2PO_4 (pH=2.2) and 20% methanol. The flow rate was maintained at 1 mL/min, and 10 μL of the sample was injected. A symmetric peak of EGCG with no interference was observed at the retention time of 12.5 min (Figure 3.2 A). A symmetric peak was also observed for DAC at the retention time of 3.1 min (Figure 3.2 B).

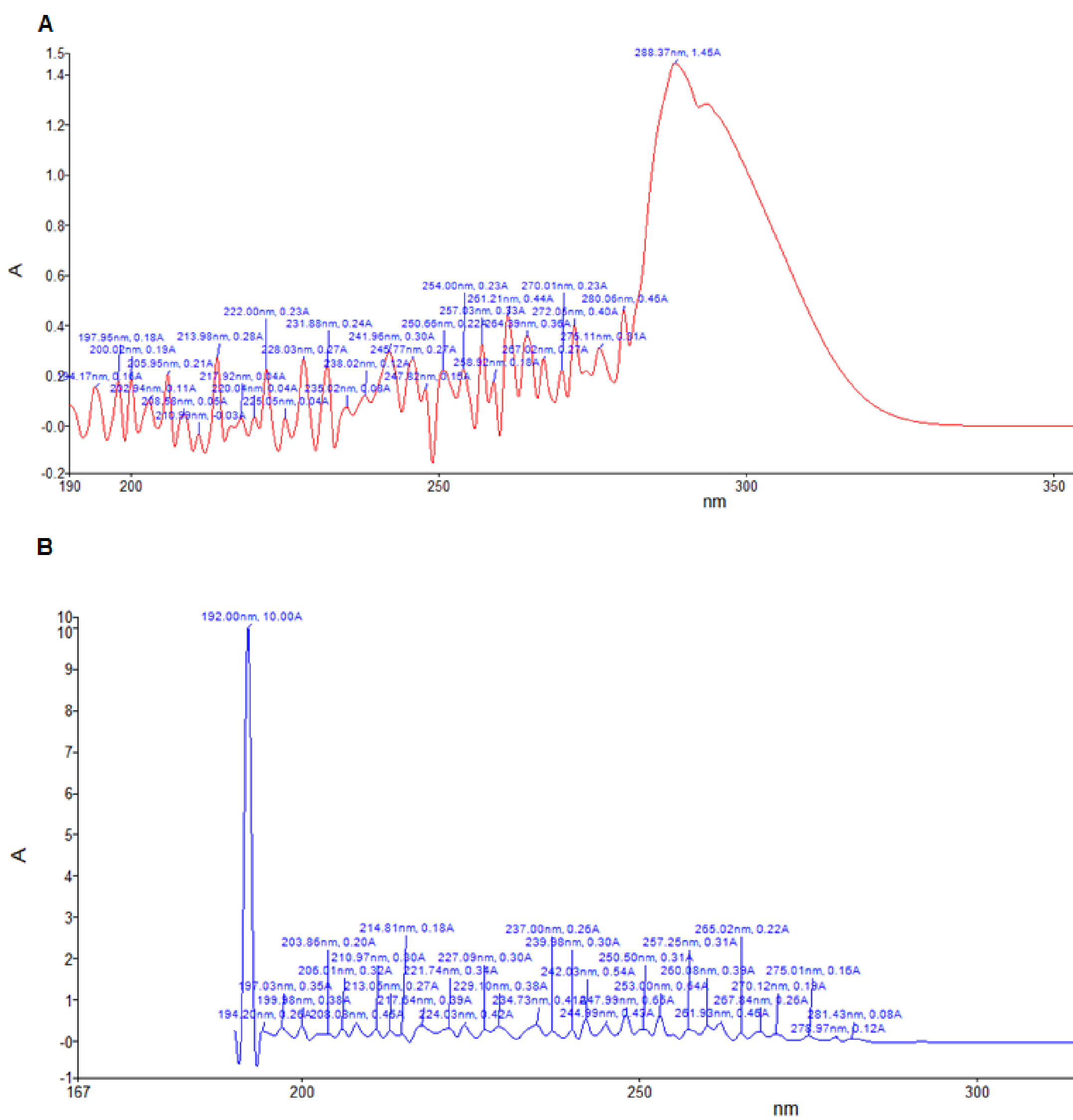
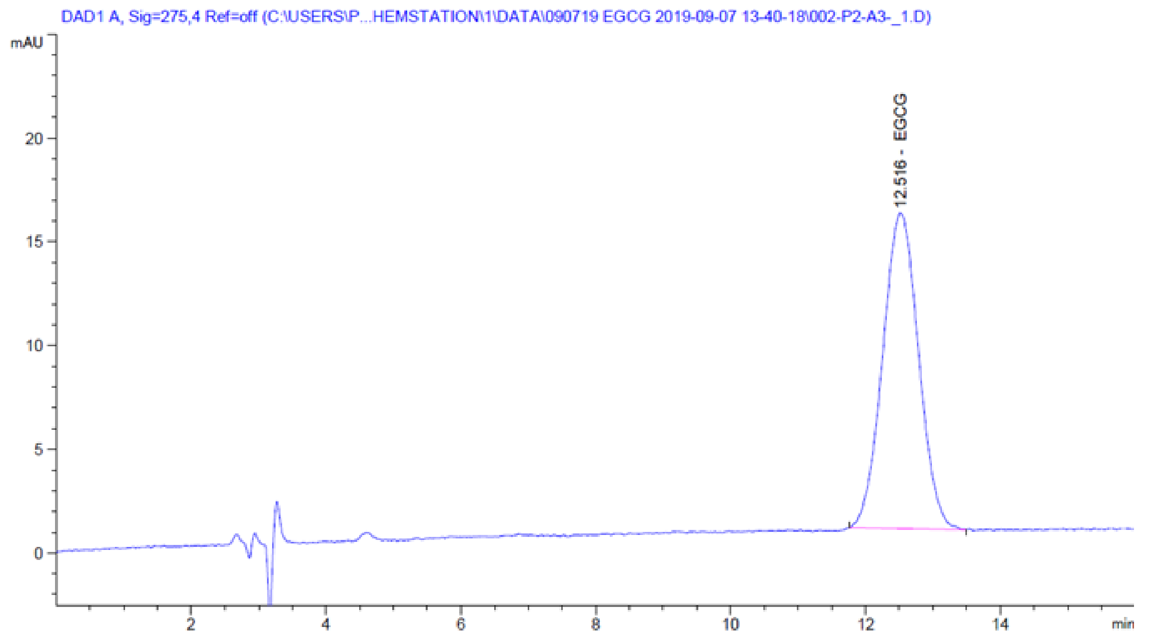


Figure 3.1 UV absorption spectrum of EGCG (A) and DAC (B).

A



B

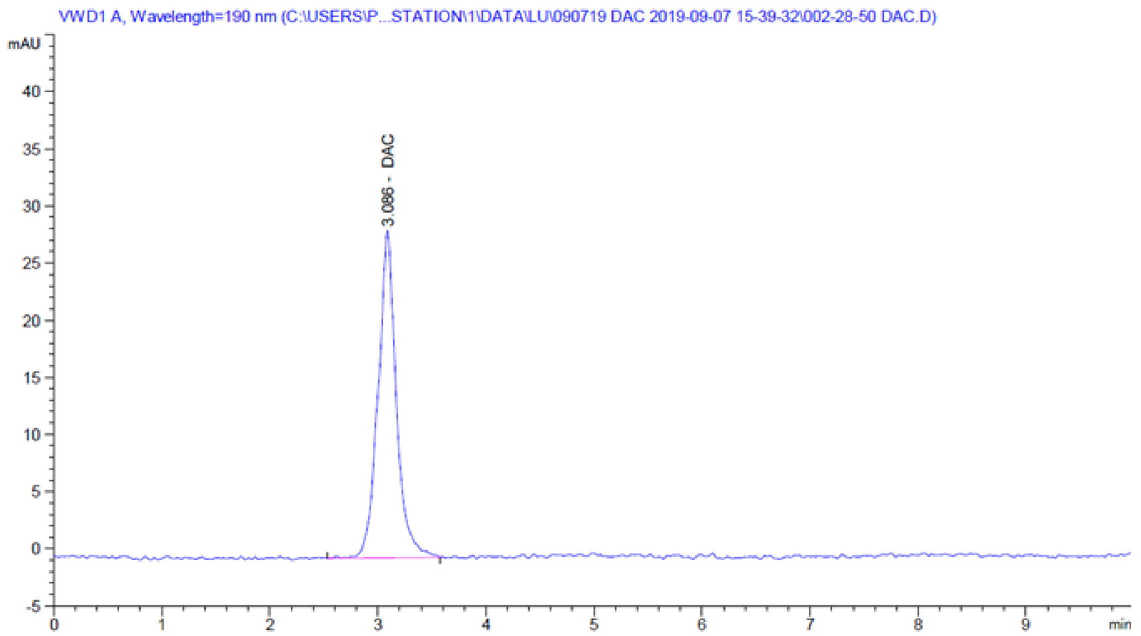
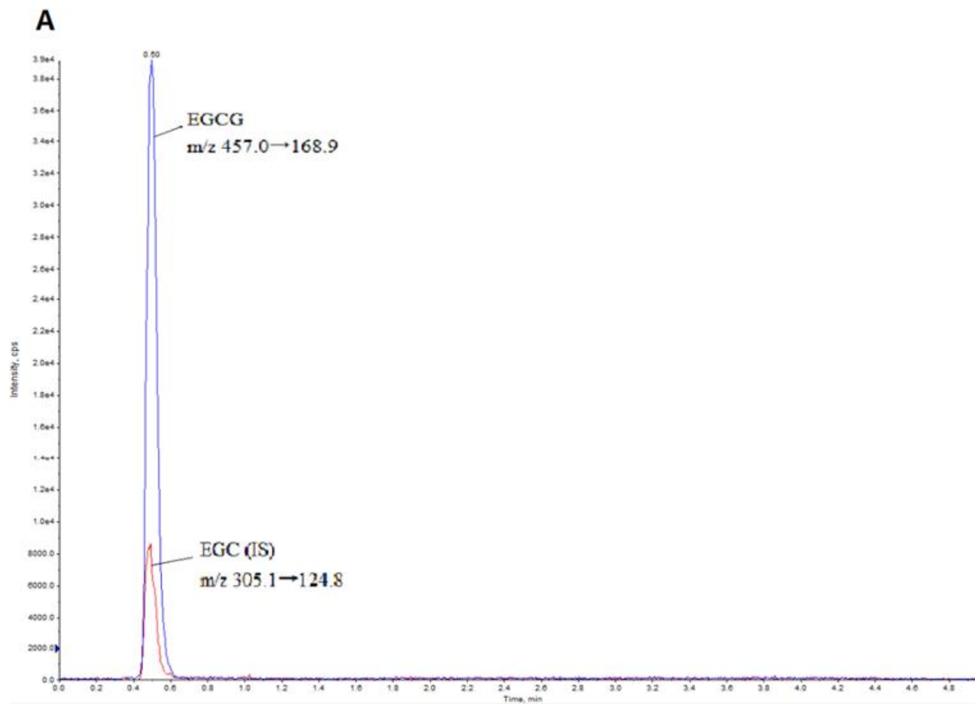


Figure 3.2 HPLC peaks for EGCG (A) and DAC (B).

However, the HPLC method is not sensitive enough for low concentrations found in biological samples. Since LC-MS/MS provides higher sensitivity and selectivity for biological samples, a LC-MS/MS method was developed. The EGCG structure contains active phenolic hydroxyl groups (Figure 1.1A) which suggests it tends to lose a proton. DAC as a nitrogen-containing heterocyclic compound (Figure 1.1B), more easily captures a proton. Also, from the the literature[9,10,11,12], EGCG and DAC are determined under negative and positive ion mode, respectively. The mass spectrometric parameters were set according to the precursor and product ion response.

3.2 Specificity and selectivity

Samples were prepared by adding working solution of EGCG, EGC, DAC and diazepam to plasma and processing by the sample preparation method. MRM chromatograms of standard drug and IS were obtained after UPLC/MS-MS analysis. As shown in Figure 3.3 and Figure 3.4, under the specified UPLC/MS-MS conditions, all substances eluted with good peak shape and high sensitivity. There was no interference from endogenous substances in blank plasma.



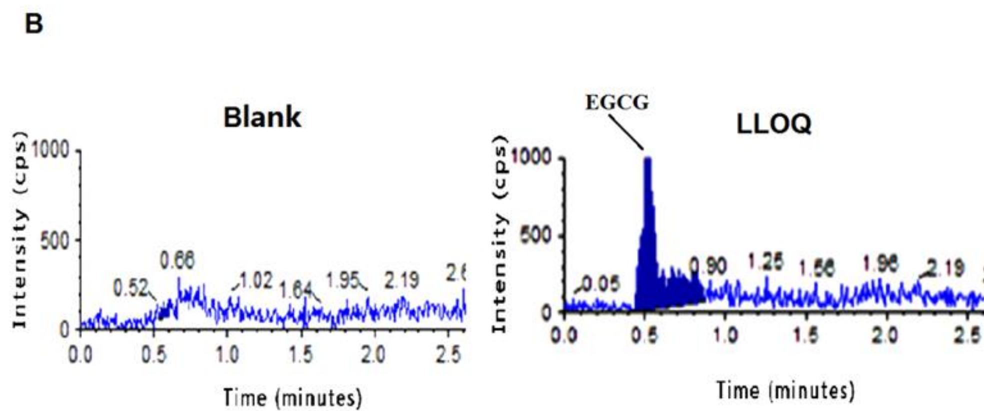


Figure 3.3 (A) MRM chromatograms of EGCG (m/z 457.0→168.9) and EGC (m/z 305.1→124.8). (B) Chromatograms of EGCG in blank plasma and LLOQ standard sample.

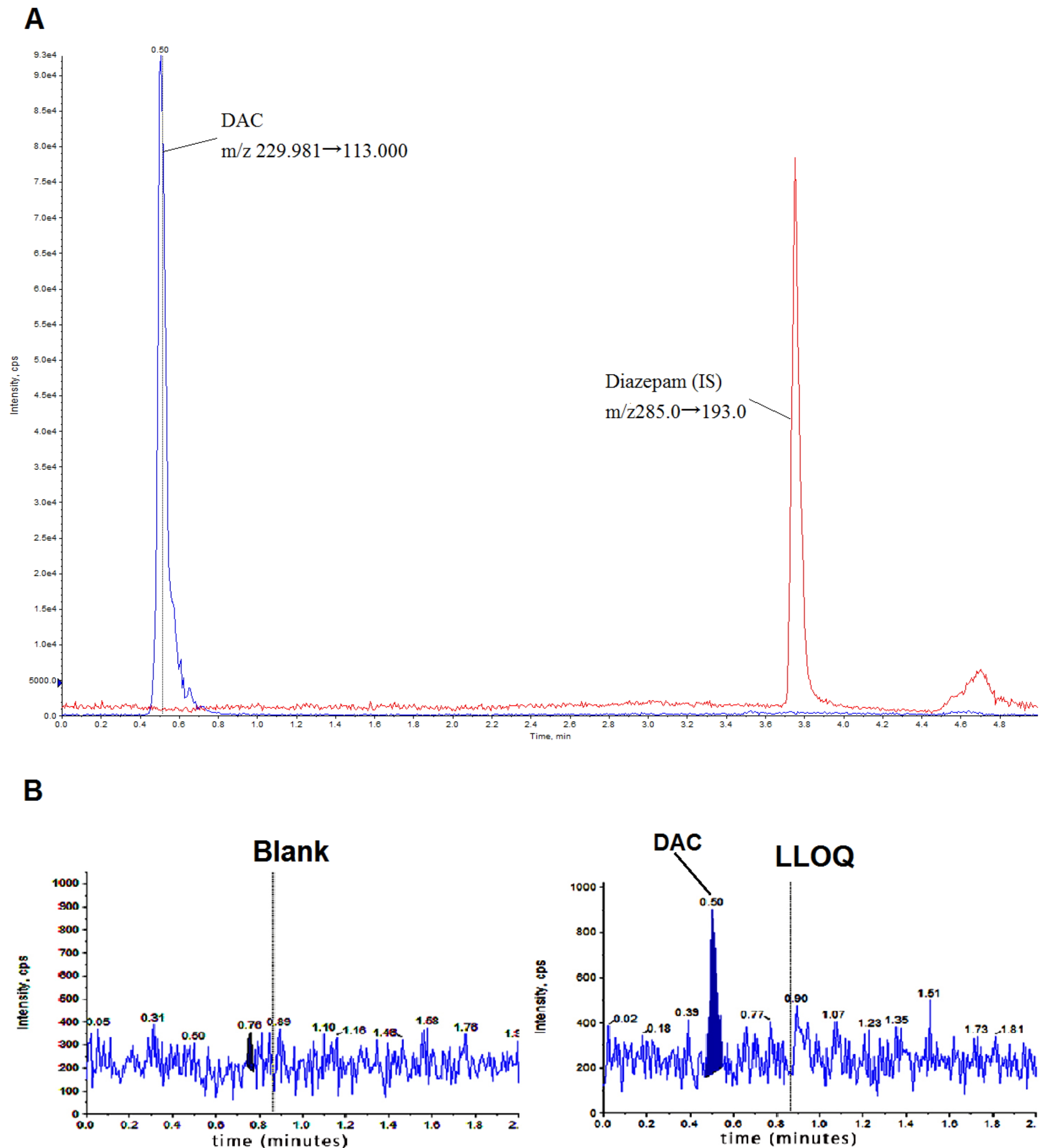


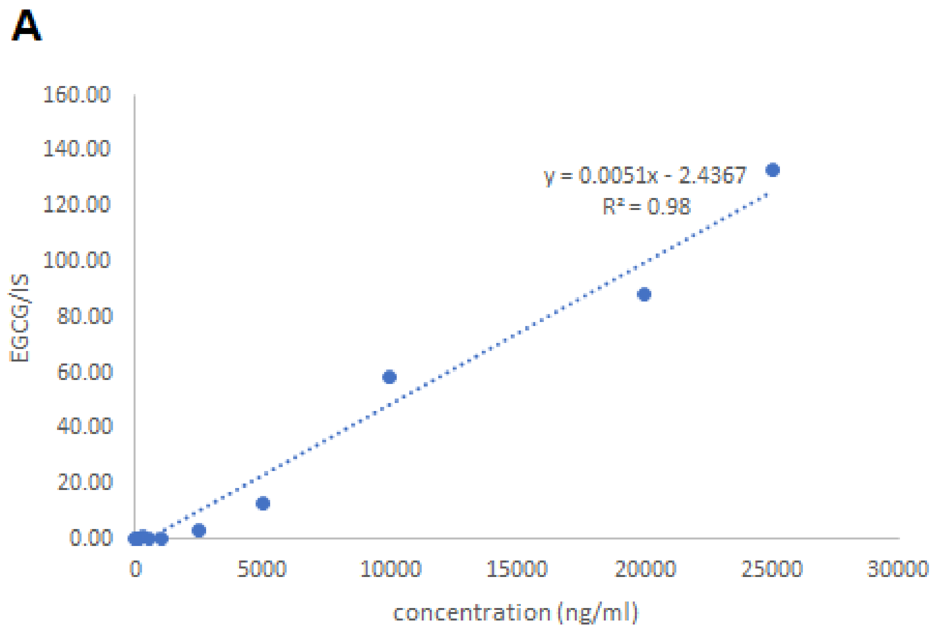
Figure 3.4 (A) MRM chromatograms of DAC (m/z 229.981→113.000) and diazepam (m/z 285.0→193.0). (B) Chromatograms of DAC in blank plasma and LLOQ standard sample.

3.3 Calibration curve and lower limit of quantification

Calibration standards were prepared by the method described in 2.4 and ranged from 10 to 25000 ng/mL (EGCG) and 10 to 10000 ng/mL (DAC). Ten calibration

standards were used for each calibration curve. Calibration curves were analyzed using least-squares regression. The regression equations of EGCG and DAC were $y = 0.0051x - 2.4367$ ($r^2 = 0.98$) and $y = 0.00001x - 0.0013$ ($r^2 = 1.0$), respectively. Where y was the ratio of standard peak area to IS peak area and x was plasma concentration (Figure 3.5).

To assess the lower limit of quantification, the signal in blank plasma was compared to signal at LLOQ. The signal at LLOQ was 5 times higher than signal in blank plasma (Figure 3.3 and Figure 3.4). The LLOQ was 10ng/mL for both EGCG and DAC.



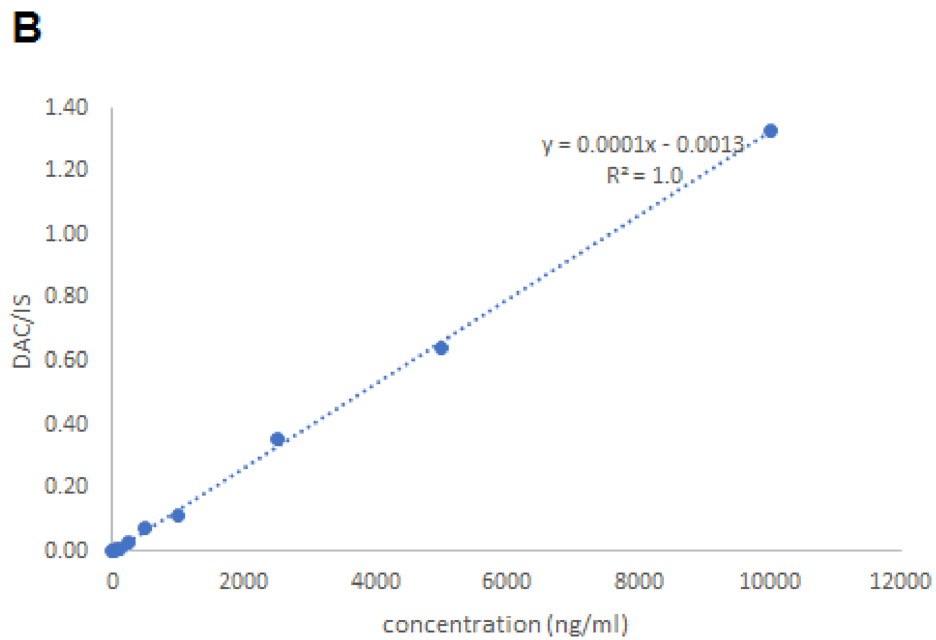


Figure 3.5 Calibration curves for EGCG (A) and DAC (B). Y axis was the ratio of standard peak area to IS peak area and x axis was plasma concentration.

All figures in chapter 3 are based on my own experimental results and analysis.

Chapter 4: Disscusion

The advantage of this this study is that UPLC-MS/MS provides a rapid determination that significantly decreases time of analysis, which is suitable for biological sample analysis.

During the experiment, the influence of different methods on the extraction of drugs in plasma samples was investigated. Methanol or acetonitrile were used for protein precipitation. Ethyl acetate was used for liquid-liquid extraction. Recovery for DAC was high using all three solvents while EGCG had low recovery after protein precipitation with methanol or acetonitrile. So ethyl acetate was used in sample preparation. Literature [10] also suggests solid phase extraction can achieve high recovery rate for EGCG.

The calibration curve for EGCG was $y = 0.0051x – 2.4367$ ($r^2 = 0.9776$). The low r^2 value may attribute to instability of EGCG. EGCG is the major bioactive catechin compound in tea. This kind of polyhydroxy compound can easily degrade. The main causes of EGCG instability are its auto-oxidation and epimerization [13,14]. Ascorbic acid was shown to stabilize EGCG [15,16]. However, our results showed a large fluctuation of peak area of EGCG samples prepared with the co-addition of 20%, 30%, 40% or 50% ascorbic acid in different batches, which made the experiment difficult to replicate. The reason may because EGCG concentration, pH, temperature and oxygen partial pressure can all effect EGCG stability [17]. In the process of sample preparation, different degrees of degradation might be produced according to different experimental

conditions. It is essential to find an effective antioxidant and control the environmental factors to reduce the effect of drug degradation on the experiment.

This method still needs further improvement and validation of recovery rate, accuracy and precision. Accuracy values (expressed as the bias) and precision values (expressed as the coefficient of variation (CV)) should be within ± 15% of the nominal concentrations, or ± 20% of LLOQ. Recovery rate (expressed as CV) should be within ± 15%. Enhancing stability of drugs and improving accuracy of the assay will be a future direction. Finally, the method should be established that the analytes show a good linear relationship within the concentration range of detection with high precision, good stability and high extraction rate. An improved version of the method could be applied to the analysis of biological samples, provides a reference for studying the EGCG/DAC combination pharmacokinetic characteristics.

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