

DOCTORAL THESIS

Development and application of liquid chromatography and electrospray-ionization mass spectrometry methods for herbal medicine analysis and for the studies of metabolism, DNA adducts and metabonomics of aristolochic acids

Chan, Wan

Date of Award:
2007

[Link to publication](#)

General rights

Copyright and intellectual property rights for the publications made accessible in HKBU Scholars are retained by the authors and/or other copyright owners. In addition to the restrictions prescribed by the Copyright Ordinance of Hong Kong, all users and readers must also observe the following terms of use:

- Users may download and print one copy of any publication from HKBU Scholars for the purpose of private study or research
- Users cannot further distribute the material or use it for any profit-making activity or commercial gain
- To share publications in HKBU Scholars with others, users are welcome to freely distribute the permanent URL assigned to the publication

**Development and Application of Liquid Chromatography and
Electrospray-Ionization Mass Spectrometry Methods for Herbal
Medicine Analysis and for the Studies of Metabolism, DNA
Adducts and Metabonomics of Aristolochic Acids**

CHAN Wan

A thesis submitted in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Principal Supervisor: Prof. CAI Zongwei

Hong Kong Baptist University

December, 2007

Abstract

Aristolochic acid (AA) is a mixture of structurally related nitrophenanthrene carboxylic acids derived from herbal species *Aristolochia* and *Asarum*. Major components of AA include AAI and AAI. This thesis focuses on the application of liquid chromatography and mass spectrometry for the herbal medicine analysis and for the studies of metabolism, DNA adducts and metabonomics of nephrotoxic and carcinogenic AA.

For the high nephrotoxicity and carcinogenicity of AA, identification of AA containing herbs is important. We described in the present thesis, a new, sensitive and selective HPLC method with fluorescence detector (HPLC-FLD) for the determination of AA in herbal medicines by using pre-column derivatization with zinc powder in acetic acid. The detection limits of developed method were 2-4 orders of magnitude lower than those from existing methods. The HPLC-FLD method has been applied for the determination of AA in herbal medicines.

Drug metabolism study is important for understanding the pharmaceutical or toxicological effect of a drug. In this study, *in vitro* and *in vivo* metabolism studies were conducted so as to gain knowledge about the metabolism and metabolic activation of AA. *In vitro* study was performed by the incubation of AA with rat liver S9, under aerobic or anaerobic conditions. The *in vivo* phase I and phase II metabolism of AA were studied by using Sprague-Dawley rats as the animal model. Structure elucidation of the metabolites was performed by using liquid chromatography coupled with electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS).

The formation of DNA adducts is the first step of chemical carcinogenesis. In this study, the feasibility of DNA aristolochic acid (DNA-AA) adducts analysis by

LC-ESI-MS was explored. When DNA was incubated with AA *in vitro*, dG-AAI, dG-AAII, dA-AAI, dA-AAII, dC-AAI and dC-AAII were detected and characterized. The developed LC-MS method was also applied to the identification and quantification of DNA-AA adducts in the kidney and liver DNA samples from the AA dosed rats. dA-AAII was found to be the most abundant among the detected DNA-AA adducts, such as dA-AAII, dA-AAI and dC-AAII. The dC-AA adducts were identified in the *in vitro* and *in vivo* systems, for the first time.

DNA binding assays demonstrated that carcinogens show site- and sequence-specificity and the biological consequence is defined by the nature of binding as well as their position in the genome. In this study, ESI-MS/MS was applied for the identification and position mapping of the DNA-AA adducts in oligonucleotides (ONDs). The observation of the modified bases (modified adenine and guanine) together with the complementary product ions ($[a_n-B*_n]^+$, w^-) from the cleavage of the 3' C-O bond adjacent to the modified base in MS/MS analyses readily enabled identification of the AA binding site in ONDs.

The toxic effects of AA to the rats were also investigated by LC-ESI-MS using the metabonomics approach. Analysis of the urine and plasma samples revealed distinct changes in the biochemical patterns in the AA dosed rats. After peak finding and alignment, principal component analysis and partial least squares discriminant analysis were used for multivariate data analysis. Potential biomarkers were studied by high-resolution MS and MS/MS analyses. Citric acid and a glucuronide-containing metabolite were observed as potential biomarkers in rat urine. A significant increase in plasma creatinine concentration was also observed in the AA dosed rats, which indicated that AA induced an adverse effect on the renal clearance function of the animals.

Table of Contents

Declaration	i
Abstract	ii
Acknowledgements	iv
Tables of Contents	vi
List of Tables	xii
List of Figures	xiii
List of Abbreviations	xx
Chapter 1 Introduction	1
1.1 Background	1
1.2 Analysis of Aristolochic Acids in Herbal Medicines	5
1.3 Metabolism of Aristolochic Acids	8
1.4 Studies of the DNA Adducts Induced by Aristolochic Acids ...	15
1.5 Identification and Position Mapping of the DNA Adducts Induced by Aristolochic Acids in Oligonucleotides	22
1.6 Metabonomic Studies of the Toxic Effects Induced by Aristolochic Acids	24
1.7 Aims of the Project	26

Chapter 2	Development and Application of a High-Performance Liquid Chromatography Fluorescence Detection Method for the Determination of Aristolochic Acids in Herbal Medicines	28
2.1	Introduction	28
2.2	Experimental	31
2.2.1	<i>Chemicals</i>	31
2.2.2	<i>Preparation of Herbal Plant Samples</i>	31
2.2.3	<i>Apparatus and Conditions</i>	34
	<i>2.2.3.1 UV Absorption and Fluorescence Spectrometer.....</i>	34
	<i>2.2.3.2 HPLC-FLD</i>	34
	<i>2.2.3.2.1 Sample Analysis</i>	34
	<i>2.2.3.2.2 Calibration and Method Validation</i>	34
	<i>2.2.3.3 HPLC-MS/MS Analysis for Method Comparison</i>	35
2.3	Results and Discussion	37
2.3.1	<i>UV Absorption and Fluorescence Spectra</i>	37
2.3.2	<i>Optimization of Derivatization Reaction Parameters</i>	41
2.3.3	<i>Recovery, Linearity, Precision, Accuracy and Detection Limit of HPLC-FLD Method</i>	42
2.3.4	<i>Application in Herbal Medicine Analysis</i>	50
2.3.5	<i>Analysis of AA by HPLC-MS/MS for Method Comparison</i>	53
2.4	Chapter Summary	56

Chapter 3	Application of Liquid Chromatography-Electrospray Ionization Mass Spectrometry for the Metabolism Studies of Aristolochic Acids	57
3.1	Introduction	57
3.2	Experimental	59
3.2.1	<i>Chemicals</i>	<i>59</i>
3.2.2	<i>Rat Liver S9 Fraction Preparation</i>	<i>59</i>
3.2.3	<i>In Vitro Incubations and Sample Preparation</i>	<i>59</i>
3.2.4	<i>In Vivo Study</i>	<i>60</i>
3.2.5	<i>LC-ESI-MS Analysis</i>	<i>61</i>
3.2.6	<i>Theoretical Calculations</i>	<i>62</i>
3.3	Results and Discussion	63
3.3.1	<i>In Vitro Metabolic Stability and Metabolite Identification</i>	<i>63</i>
3.3.2	<i>In Vivo Metabolism Study of AA</i>	<i>69</i>
3.3.3	<i>Investigation of the Reductive Activation of AA</i>	<i>85</i>
3.4	Chapter Summary	93
Chapter 4	Application of Liquid-Chromatography and Mass Spectrometry for Studying the DNA Adducts Induced by Aristolochic Acids	94
4.1	Introduction	94
4.2	Experimental	98

4.2.1	<i>Chemicals</i>	98
4.2.2	<i>Preparative Scale Synthesis of the Deoxyadenosine -Aristolochic Acid Adducts</i>	98
4.2.3	<i>In Vitro Studies</i>	99
4.2.3.1	<i>In Vitro Incubation with Zinc</i>	99
4.2.3.2	<i>In Vitro Incubation with Xanthine Oxidase</i>	100
4.2.4	<i>In Vivo Study</i>	100
4.2.4.1	<i>Animal Experiment</i>	100
4.2.4.2	<i>DNA Isolation</i>	100
4.2.5	<i>DNA Digestion and Adduct Enrichment</i>	101
4.2.6	<i>Quantitative Analysis of DNA-AA Adducts in Rat Kidney and Liver</i>	101
4.2.7	<i>LC-ESI-MS Analysis</i>	103
4.3	Results and Discussion	104
4.3.1	<i>Preparation, Isolation and Identification of 7-(deoxyadenosine-N⁶-yl)-aristolactam II (dA-AAII)</i>	104
4.3.2	<i>Optimization of the HPLC and ESI-MS Parameters</i>	108
4.3.3	<i>Analysis of DNA-AA Adducts Formed in in Vitro Activation Systems</i>	110
4.3.4	<i>In Vivo Study</i>	122
4.4	Chapter Summary	133

Chapter 5	Identification and Position Mapping of the DNA Adducts Induced by Aristolochic Acids in Oligonucleotides by Electrospray-Ionization Tandem-Mass Spectrometry	134
5.1	Introduction	134
5.2	Experimental	136
5.2.1	<i>Reagents</i>	136
5.2.2	<i>Oligonucleotide Modification and Purification</i>	136
5.2.3	<i>Methods and Instrumentation</i>	137
5.3	Results and Discussion	138
5.3.1	<i>Optimization of ESI-MS Parameters</i>	138
5.3.2	<i>Preparation of Modified Oligonucleotides</i>	138
5.3.3	<i>ESI-MS and ESI-MS/MS Analyses of the Modified and Unmodified Oligonucleotides</i>	139
5.4	Chapter Summary	157
Chapter 6	Metabonomics Investigation of the Biochemical Effects Induced by Aristolochic Acids	158
6.1	Introduction	158
6.2	Experimental	160
6.2.1	<i>Chemicals</i>	160
6.2.2	<i>Animal Experiment and Sample Collection</i>	160
6.2.3	<i>Sample Preparation</i>	161

6.2.4	Instrumentation	161
6.2.4.1	<i>Chromatography</i>	161
6.2.4.2	<i>Mass spectrometry</i>	161
6.2.5	Data Analysis	163
6.3	Results and Discussion	164
6.3.1	<i>Laboratory Parameters from Animal Experiment</i>	164
6.3.2	<i>Creatinine Analysis</i>	168
6.3.3	<i>Metabolic Profiling of Rat Urine by LC-MS Analysis</i>	171
6.3.4	<i>LC-MS/MS for Structural Elucidation of the Potential Biomarkers</i>	181
6.4	Chapter Summary	184
Chapter 7	Conclusion	185
Chapter 8	List of References	188
	Curriculum Vitae	204
	Outcome of the Thesis Work	205
	A. Publications	205
	B. Conference and Symposium Presented	206
	C. Scholarship	207