Chromatographic Method Combined with Bioassay for Rapid Screening of Enzyme Inhibitors from Herbal Extracts

by

LI De-Qiang

Doctor of Philosophy in Biomedical Sciences

2013

Institute of Chinese Medical Sciences

University of Macau

UNIVERSIDADE DE MACAU
仁義禮知信
澳門大學

Chromatographic Method Combined with Bioassay for Rapid Screening of Enzyme Inhibitors from Herbal Extracts

by

LI De-Qiang

Supervisor: Professor LI Shao-Ping

Co-Supervisor: Professor Zhang Lan-Tong

Doctor of Philosophy in Biomedical Sciences

2013

Institute of Chinese Medical Sciences

University of Macau

UNIVERSIDADE DE MACAU
仁義禮知信
澳門大學

Author's right 2013 by
LI, De-Qiang



UNIVERSIDADE DE MACAU
仁義禮知信
澳門大學

Acknowledgements

I would like to thank all the people who have helped and inspired me during my doctoral study.

First of all, I would like to express my deep and sincere gratitude to my supervisor, Prof. Shao-Ping Li. His intelligent mind, diligent and rigorous academic attitude enlightened my whole research work. Also, I would like to thank Prof. Yi-Tao Wang, the director of our institute, and Prof. Lan-Tong Zhang from Hebei Medical University, for helping me overcome the difficulties in studying.

I especially want to thank Prof. Qing-Wen Zhang, Prof. Ying Zheng and Prof. Ru Yan from Institute of Chinese Medical Sciences for their help in my research work. I am deeply grateful to Dr. Jing Zhao for her constructive comments, constant supervision and encouragement.

I owe my most sincere gratitude to all staff of administrative office (Hattie, Ada, Chloe, Leon and Sandy) for their help during my project. I, likewise, want to thank my friends and colleagues, Chun-Feng Qiao, Yuan-Jia Hu, Feng-Qing Yang, Jia Guan, Zheng-Ming Qian, Kun Feng, Xiao-Jia Chen, Wei-Hua Huang, Jing Li, Lan-Zhen Meng as well as other classmates for offering me help, and giving me the great memory of the happiness to study and work together.

Last but not least, my gratitude also extends to my parents, my wife and my daughter for their full assisting, supporting and caring.

The research was supported by grants from University of Macau (UL015A).

Abstract

Natural products, which have been recognized as the most productive source for discovery of new molecular entities, are getting more and more attention for drug discovery. In the research work, high performance liquid chromatography (HPLC) combined with bioassay was used to rapidly discover bioactive compounds from Chinese herbs on the basis of important targets towards to diabetes II (*i.e.* α-glucosidase) and gout (*i.e.* xanthine oxidase). Two on-line screening platforms based on post-column bioactivity assay and pre-column affinity assay were developed, respectively.

For bioactivity-based screening, some hyphenated techniques coupling HPLC to post-column bioassay were developed to screen and identify the bioactive compounds from complex mixtures without the need of cumbersome purification and subsequent biochemical measurement. For α-glucosidase, three selected beverages were firstly screened by the on-line HPLC-DAD-MS/MS analysis and two potential active compounds with inhibitory activity from Pu-erh tea, EGCG and ECG, were successfully found. Considering the possible false positive inhibition derived from non-specific denatured enzyme, an improved method was subsequently developed with integration of sample preparation and the on-line analysis, and false inhibition caused by tannin greatly decreased. Using this method, two compounds, *i.e.*, corilagin and ellagic acid, with specific α-glucosidase inhibition, were discovered. For xanthine oxidase (XO), two inhibitors including baicalein and wogonin from *Scutellaria baicalensis* extract were found by on-line fluorescence-based biochemical detection. On account of the XO reaction *in vivo*, a post-column dual-bioactivity assay was established and applied to measuring the XO inhibitory and free radical scavenging activities of individual compounds in *Oroxylum indicum*

extract.

For affinity-based screening, an automated on-line restricted-access material solid-phase extraction coupled to column-switching high-performance liquid chromatography (on-line RAM SPE-HPLC) system combined with off-line functional assay was developed for the XO inhibitors screening. The natural product, *Oroxylum indicum* extract, was analyzed by the developed on-line system, and four compounds with XO inhibition were found and their relative binding capabilities were also ranked.

In summary, the two established on-line bioactive screening platforms have been proved to be very useful for rapid profiling and identification of individual active components in complex mixtures, which provided powerful tools for natural product-based drug discovery.

UNIVERSIDADE DE MACAU
仁義禮知信
澳門大學

Declaration

I declare that the thesis here submitted is original except for the source materials explicitly acknowledged and that this thesis as a whole, or any part of this thesis has not been previously submitted for the same degree or for a different degree.

I also acknowledge that I have read and understood the Rules on Handling Student Academic Dishonesty and the Regulations of the Student Discipline of the University of Macau.

UNIVERSIDADE DE MACAU
仁義禮知信
澳門大學

Table of Contents

Acknowledgements ..... **i**
Abstract ..... **ii**
Declaration ..... **iv**
List of Tables and Figures ..... **vii**
List of Abbreviations ..... **x**
Chapter 1 Introduction **1**
1.1 General Background **1**
1.2 Specific Background **2**
1.2.1 Solution-based affinity screening methods 3
1.2.2 Post-column bioactivity-based screening methods 7
1.3 Research Goals and Objectives **20**
1.4 Research Methodology and Design **21**
1.5 Potential Contributions **22**
1.6 Organization of the Thesis **22**
1.7 Statement of Originality **24**
References **28**
Chapter 2 *α*-Glucosidase inhibitors screening using high-performance liquid chromatography coupled with on-line post-column bioassay **32**
2.1 Introduction **32**
2.2 Chemicals, reagents and materials **33**
2.3 *α*-Glucosidase inhibitors screening from three selected beverage extracts **34**
2.3.1 Experimental 35
2.3.2 Results and discussion 38
2.4 Specific *α*-glucosidase inhibitors screening from Chinese herbs **44**
2.4.1 Experimental 45
2.4.2 Results and discussion 48
2.5 Conclusion **57**
References **57**
Chapter 3 Xanthine oxidase inhibitors screening by high performance liquid chromatography combined with bioassay **62**
3.1 Introduction **62**
3.2 Chemicals, reagents and materials **63**
3.3 Screening of xanthine oxidase inhibitors using on-line HPLC coupled with post-column fluorescence-based biochemical detection **64**
3.3.1 Experimental 65
3.3.2 Results and discussion 69
3.4 Simultaneous screening of xanthine oxidase inhibitors and free radical scavengers from complex mixture by on-line HPLC coupled with post-column dual-bioactivity assay **78**
3.4.1 Experimental 79
3.4.2 Result and discussion 82
3.5 Screening xanthine oxidase inhibitors from complex mixture by on-line restricted-access material solid-phase extraction coupled to column-switching HPLC **93**
3.5.1 Experimental 96
3.5.2 Results and discussion 100
3.6 Conclusion **107**

References **108**
Chapter 4 Conclusions **113**
4.1 Conclusions **113**
4.2 Limitations of Current Study **116**
4.3 Perspectives for Future Work **117**
References **118**
Appendices **120**
Curriculum Vitae **123**

UNIVERSIDADE DE MACAU
仁義禮知信
澳門大學

List of Tables and Figures

Table 1.1 Summary of the applications of on-line post-column function-based screening 8
Table 2.1 Inhibition of selected three beverage extracts on α-glucosidase (n=3) 38
Table 2.2 Summarized contents of EGCG and ECG in different teas 44
Table 2.3 The chromatographic conditions for extracts separation 48
Table 2.4 α-Glucosidase inhibitory properties of most effective extracts of Chinese medicines 49
Table 2.5 The retention time, UV and MS characteristics of the main active peaks in extracts 53
Table 3.1 The chromatographic condition for sample separation 69
Table 3.2 Inhibition of positive drug and *Scutellaria baicalensis* extract on xanthine oxidase by off-line assay (n=3) 75
Table 3.3 The chromatographic condition for sample separation 88
Table 3.4 The retention times of four reference compounds in the on-line HPLC dual activities detection system 90
Table 3.5 Retention time, maximum absorbance and mass spectrum of the main active peaks in *Oroxylum indicum* extract 92
Table 3.6 Elution programs of on-line RAM SPE-HPLC analysis 98
Table 3.7 Binding degree and inhibitory activity against xanthine oxidase 107
Fig. 1.1 The common process for bioactive compounds discovery using the pre-column solution-based target affinity screening. 4
Fig. 1.2 The scheme of on-line post-column bioactivity-based screening system. (E = enzyme source/receptor; S = substrate) 10
Fig. 1.3 Hyphenation of the BCD and the RP gradient separation using the developed countergradient system. 14
Fig. 1.4 Scheme of high resolution screening assay for enzyme inhibitors with high-temperature LC. 15
Fig. 1.5 On-line HPLC–SFEID (segmented flow enzyme inhibition detector) system for acetylcholinesterase inhibition assay. 16
Fig. 1.6 Comparison of on-line HPLC–EID and HPLC–SFEID with HPLC–UV. 17
Fig. 1.7 The microfluidic chip as used for bioactivity screening. 18
Fig. 1.8 Schematic view of the triple and parallelized CYP1A, 2B, and 3A EAD system in gradient HPLC mode. 19
Fig. 2.1 Diagrammatic scheme of HPLC-DAD-MS-BCD for rapid screening and identification of α-glucosidase inhibitors. 37
Fig. 2.2 The effects of organic modifiers on enzyme activity. 39
Fig. 2.3 The typical UV chromatograms before (DAD) and after (BCD) on-line biochemical assay of acarbose (A), Pu-erh tea (B), Eagle tea (C) and Radix Glycyrrhizae (D) extracts, and mixed solution (E) of EGCG and ECG. 41
Fig. 2.4 UV, MS and MS_2 spectra and fragments illustration of peak 1 (left) and peak 2 (right). 42
Fig. 2.5 Inhibitory effects (A) and Lineweaver–Burk plots of EGCG (B), ECG (C) and acarbose (D) on α-glucosidase. 43
Fig. 2.6 UV chromatogram at 280 nm and biochemical response chromatogram at 405 nm of 1.5 mM EGCG (**1**) and 1.8 mM ECG (**2**). 50
Fig. 2.7 The typical UV and on-line biochemical assay chromatograms of some extracts from fruit of *Terminalia chebula* (A), flowers of *Rosa rugosa* (B) and *Eugenia caryophyllata* (C) and husk of *Punica granatum* (D) before

(left) and after (right) gelatin precipitation. E, extract; E+G, extract treated with gelatin 52
Fig. 2.8 The inhibitory effects of corilagin (A) and ellagic acid (B) on α-glucosidase activity and the kinetic properties of *α*-glucosidase in the absence and presence of corilagin (C) or ellagic acid (D) in Lineweaver-Burk plots using different concentrations of substrates. 56
Fig. 3.1 Schematic representation of the continuous xanthine oxidase assay (A) and on-line HPLC-DAD-BCD assay for screening of XO inhibitors (B). 68
Fig. 3.2 The fluorescence excitation (A) and emission (B) spectrum of substrate and product. ♦ Substrate; ■ Product. 70
Fig. 3.3 Enzyme assay optimization. (A) Effect of pH on the enzyme reaction. (B) Enzyme concentration response. (C) Time course of the enzyme reaction at room temperature. (D) Enzyme kinetics. (E) Influence of organic modifiers on enzymatic activity. ◆ DMSO; ▲ MeOH; ■ ACN. 72
Fig. 3.4 (A) Inhibition of allopurinol by continuous xanthine oxidase assay at different concentrations. (B) The observed dose-response plot of continuous xanthine oxidase assay for allopurinol was compared to the result of off-line fluorescence assay. 74
Fig. 3.5 Typical chromatograms of positive drug (A) and a complex mixture of *Scutellaria baicalensis* extract (B, red: 40 μL; blue: 20μL) applying the developed on-line HPLC-DAD-BCD-MS system. 76
Fig. 3.6 UV, MS and MS_2 spectra and structures of peak 1' (left) and peak 2' (right) 77
Fig. 3.7 Scheme of the analytical system for screening of XO inhibitors and free radical scavengers from complex mixture by hyphenation of the post-column dual-bioactivity assay to the RP gradient separation. 82
Fig. 3.8 UV-spectrums of xanthine oxidase (0.05 U/mL), xanthine (0.25 mM), uric acid (0.20 mM) and H_2O_2 (0.25 mM). 84
Fig. 3.9 Effects of (A) substrate concentration, (B) enzyme concentration, (C) reaction time and (D) organic solvent (ACN) on the XO activity by microplate-based HPLC assay. 86
Fig. 3.10 (A) Inhibition of allopurinol by FIA-BCD analysis at different concentrations. (B) The observed dose-response plot of FIA-BCD for allopurinaol was compared to the result of microplate-base HPLC assay. .. 87
Fig. 3.11 Typical chromatograms of a mixture of reference standards (Peak 1: allopurinol, peak 2: rutin, peak 3: quercetin and peak 4: baicalein) applying the developed on-line HPLC coupled with post-column dual-bioassay system. (A) BCD signals of the XO inhibition of separated mixture (a) and its negative control (native-UV detection) (b) at 295 nm. (B) The typical UV-chromatogram at 265 nm (c) and ABTS assay profile (d) (750 nm, free radicals scavenger detected as negative peaks) of mixed standards. (C) Total ion chromatogram (TIC) of HPLC-ESI(-)-MS separation of mixed reference standards. 90
Fig. 3.12 Typical chromatograms of *Oroxylum indicum* extract applying the developed on-line HPLC coupled with post-column dual-bioassay system. (A) BCD signals of the XO inhibition of extract (a) and its negative control (native-UV detection) (b) at 295 nm. (B) The typical UV-chromatogram at 265 nm (c) and ABTS assay profile (d) (750 nm, free radicals scavenger detected as negative peaks) of extact. (C) Total ion chromatogram (TIC) of HPLC-ESI(-)-MS separation of *Oroxylum indicum* extract. 91

Fig. 3.13 The structures of identified bioactive compounds with xanthine oxidase inhibitory and/or free radicals scavengering activities..... 92
Fig. 3.14 Scheme of on-line RAM SPE-HPLC assay for screening of xanthine oxdiase inhibitors.. 98
Fig. 3.15 The chromatograms of single RAM SPE column analysis of xanthine oxidasse (A), mixed standards (B) (green line: 0.20 mL/min; red line: 0.15 mL/min; blue line: 0.10 mL/min) and MEOS (C). 102
Fig. 3.16 UV chromatograms of mixed references incubated with buffer (blue line) and denatured xanthine oxidase (red line), respectively. 104
Fig. 3.17 UV chromatograms of on-line RAM SPE-HPLC analysis for binding selectivity of mixed standards (A, 330 nm) and MEOS (B, 270 nm)..... 104
Fig. 3.18 UV chromatograms (270 nm) of on-line RAM SPE-HPLC analysis for a competitive binding test between MEOS and the known ligand allopurinol. 105
Fig. APP-1 The MS spectras of main compounds in *Oroxylum indicum* extract 120
Fig. APP-2 The UV and TIC chromatograms of *Oroxylum indicum* extract 122

UNIVERSIDADE DE MACAU
仁義禮知信
澳門大學

List of Abbreviations

2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt	ABTS
acetylcholinesterase	AChE
acetonitrile	ACN
acetylcholine binding protein	AChBP
angiotensin-converting enzyme	ACE
atmospheric pressure chemical ionization	APCI
capillary high performance liquid chromatography	CHPLC
Chinese medicines	CMs
cyclooxygenase	COX
cytochrome P450	Cyto P450
dihydrofolate reductase	DHFR
diode array detector	DAD
fluorescence detector	FLD
flow injection analysis	FIA
electrospray ionization	ESI
enzyme inhibition detection	EID
estrogen receptor	ER
fourier transform ion cyclotron resonance mass spectrometry	FTICR-MS
frontal affinity chromatography	FAC
glutathione-S-Transferase	GST
high performance liquid chromatography	HPLC
high temperature liquid chromatography	HTLC
high-throughput screening	HTS
high-resolution screening	HRS
human estrogen receptor	hER
human serum albumin	HSA
infrared	IR
ion trap time-of-flight mass spectrometry	IT-TOF-MS
laser-induced fluorescence	LIF
lightemitting diode	LED
limits of detection	LOD
liquid-liquid extraction	LLE
mass spectrometry	MS
methanol	MeOH
nuclear magnetic resonance	NMR
phosphate buffer salt solution	PBS
phosphodiesterase	PDE
preparative high performance liquid chromatography	PHPLC
p-nitrophenyl α-D-glucopyranoside	PNPG
quadrupole time-of-flight mass spectrometry	Q-TOF-MS

relative standard deviation	RSD
retention time	RT
restricted access material	RAM
segmented flow enzyme inhibition detection	SFEID
solid phase extraction	SPE
size exclusion chromatography	SEC
thin layer chromatography	TLC
turbulent flow chromatography	TFC
time-of-flight mass spectrometry	TOF-MS
traditional Chinese medicines	TCMs
two-dimensional liquid chromatography	2DLC
ultra performance liquid chromatography	UPLC
ultraviolet	UV
uric acid	UA
visible	Vis
xanthine oxiase	XO
relative standard deviation	RSD
retention time	RT

UNIVERSIDADE DE MACAU
仁義禮知信
澳門大學