Determination of contents of four alkaloids in *Pericarpium arecae* by quantitative analysis of multi-components by single-marker

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Abstract: By using a typical component in traditional Chinese medicine *Pericarpium Arecae* (PA), quantitative analysis of multi-components by single-marker (QAMS) was performed to determine the contents of four alkaloids. With a column packed with strong cation exchange bonded silica particles, the alkaloids were well separated, showing linear relationships within certain ranges. The limit of detection, limit of quantitation, precision, stability, repeatability and recovery all met requirements. By employing arecoline as internal standard, relative correction factors for arecaidine, guvacine and guvacoline at five concentrations were detected with three HPLC systems and three HPLC columns. The peaks of arecaidine, guvacine and guvacoline were positioned, during which the columns with the same packing materials from different manufacturers significantly affected relative retention values and retention time differences of the alkaloids. However, the columns, from different batches, managed to give relative retention values satisfying the requirements of HPLC peak positioning. The Thermo Fisher Scientific column packed with strong cation exchange bonded silica particles was finally selected by considering resolution and peak time. Compared with the external standard method, QAMS detected the alkaloid contents in 12 PA samples more accurately and reliably. The results provide valuable evidence for content determination and quality control of alkaloids in PA.

Keywords: *Pericarpium Arecae*; alkaloid, quantitative analysis of multi-components by single-marker.

INTRODUCTION

Pericarpium Arecae (PA), which is the dry pericarp of *Areca catechu* L., is prepared by harvesting the immature fruit, and then by boiling, drying and cutting it in half longitudinally. Originating from Southeast Asia and East Africa, PA has now been widely grown in Hainan, Yunnan, Guangxi, Guangdong and Taiwan provinces in China (Wu, 2009). PA was first recorded in "Ri Hua-zi's Materia Medica" and "Kai Bao Materia Medica", and has been used in clinical practice in China and other areas for about 1000 years. Compared with domperidone and mosapride, PA can regulate gastrointestinal motility disorders effectively and safely, without drug dependence (Wang *et al.*, 2004).

It is well-established that alkaloids in *A. catechu* such as arecoline and arecaidine exert regulatory effects on the stomach and intestine (Giri *et al.*, 2010; Voigt *et al.*, 2013; Garg *et al.*, 2014). Therefore, alkaloids in PA, such as arecoline, arecaidine, guvacine and guvacoline, may be associated with the regulation of gastrointestinal motility. The structures of the four alkaloids are shown in fig. 1.

Up to now, the quality control of PA has seldom been studied, and quantitative analysis of multi-components for the alkaloids has never been performed because standards such as arecaidine, guvacine and guvacoline were costly. Hence, by using cheap, easily available arecoline as the standard, quantitative analysis of multi-components by single-marker (QAMS) was conducted to establish a multi-indicator evaluation model for PA.

Inspired by the internal standard method, the correction factor method and the principal component self-control method, QAMS uses typical, cheap and readily available single component as the internal standard to establish relative correction factors (RCFs) with other components based on the directly proportional relationship between the component content within the linear range and the response value. Therefore, the contents of other components can be calculated with these RCFs (Gao *et al.*, 2011; Gao *et al.*, 2012). QAMS can only be carried out when the HPLC peak of sample is positioned by using the retention time difference or the relative retention value.

Currently, QAMS has been applied in the determination of complex multiple components in traditional Chinese medicine (TCM). For example, QAMS was used to simultaneously determine five alkaloids in the rhizoma of *Coptis chinensis* with a berberine marker (Kuang *et al.*, 2009), seven anthraquinone derivatives in rhubarb with an emodin marker (Gao *et al.*, 2009), three curcuminoids in the rhizome of *Curcuma longa* with a curcumin marker

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(Li *et al.*, 2014), nine ginsenosides in *Panax ginseng* and *Panax notoginseng* using a ginsenoside Rg1 marker (Zhu *et al.*, 2008) and three components in *Guanmaikang capsula* with a puerarin marker (Zou *et al.*, 2008). These applications indicate that QAMS is a good approach to determine multiple components in complex samples, especially in TCM and its preparations.

TCM multiple components have once been separated by a column packed with octadecyl-bonded silica particles, which, however, are not suitable for the separation of polar small-molecule alkaloids (e.g. arecoline) in PA. As a result, we herein measured the contents of four alkaloids in PA by QAMS with a column packed with strong cation exchange bonded silica particles.

MATERIALS AND METHODS

Reagents and apparatus

The apparatus used in this study included: Waters e2695 HPLC-Waters 2489 UV/Visible detector, Waters e2695 HPLC-Waters 2998 Photodiode Array detector, Agilent 1100 HPLC-G1315D diode array detector (Agilent, USA), Shimadzu LC-20AB HPLC-SPD-M20A diode array detector (Shimadzu, Japan),

Thermo SCX column (250mm × 4.6mm, 5μm, Thermo, USA), Ultimate XB-SCX column (250mm × 4.6 mm, 5 μm, Yuexu Materials Technology (Shanghai) Co., Ltd., China), Waters SCX column (250mm × 4.6mm, 5μm, Waters, USA),

BP211D electronic balance (Sartorius, Germany), KQ-200KDE high-power numerical control ultrasonic cleaner (Kunshan Ultrasonic Instrument Co., Ltd.), and UNIQUE-S15 laboratory water purification system.

The standards used in this study included arecoline hydrobromide (National Institutes for Food and Drug Control, batch number 111684-20040), arecaidine hydrochloride (Alfa, UK, batch number 10114762), guvacoline hydrobromide (Toronto, Canada, batch number 3-MP-45-3), guvacine hydrochloride (Tocris, UK, batch number 18 A/123897), with the mass fractions all being ≥98%. Besides, acetonitrile (HPLC grade, Tedia, USA), phosphoric acid (HPLC grade, Tianjin Kermel Chemical Reagent Co., Ltd.), ionized water and other analytical grade reagents were used.

Drugs

Twelve batches of medicinal materials collected from Guangdong, Gyuangxi, Taiwan, Yunnan and Henan provinces were identified as PA by Professor Jianwei Chen in College of Pharmacy, Nanjing University of Chinese Medicine.

Preparation of standard solutions

Arecoline hydrobromide (19.98mg), arecaidine hydrochloride (6.39mg), guvacoline hydrobromide (4.49 mg) and guvacine hydrochloride (6.50mg) were dissolved by the mobile phase, calibrated to 10 ml as the standard solutions and stored at 4°C.

Preparation of sample solution

PA powders (screened by No. 2 sieve, about 0.5g) were dissolved by 25ml of acetonitrile-0.5% phosphoric acid (30:70), added in a 50ml covered conical flask, weighed, sonicated for 20min and pH-adjusted to 3-4 by concentrated ammonia solution. After supplementing the lost solvent, the solution was shaken, from which the supernatant was collected, centrifuged at 6000 rpm for 5 min, and passed through 0.45μm micro filtration membrane. Then the filtrate was used as the sample solution.

HPLC conditions

Waters e2695 HPLC, Waters 2489 variable-wavelength UV detector and Thermo SCX column (250mm × 4.6 mm, 5μm) were used. The HPLC conditions were optimized as: Mobile phase, acetonitrile-0.1% phosphoric acid (pH was adjusted to 3.6 by concentrated ammonia solution) (73:27), gradient elution; column temperature, 30°C; detection wavelength, 215nm; flow rate, 1.0ml·min^{-1}; injection volume, 10μl. The HPLC results are shown in fig. 2.

STATISTICAL ANALYSIS

Relative standard deviation (%) = 100× (standard deviation/mean).

RESULTS

Optimization of HPLC conditions

HPLC was performed with Waters DAD detector from 200nm to 400nm. With a stable baseline when detected at 215nm, the four alkaloids all showed intense absorptions and the peaks were well separated after pH adjustment. The resolution was significantly affected by pH. At pH lower than 3.3 or from 3.6 to 3.8, the peaks of arecaidine and guvacine were symmetrical and easily separable, but those of the other two alkaloids could not be well separated. However, the four peaks were symmetrical and completely separated at pH 3.6, so a pH 3.6 acetonitrile-0.1% phosphoric acid mixture was selected as the mobile phase.

Linear range, limit of detection and limit of quantitation

The standard stock solution was diluted by the mobile phase and prepared into five concentrations to plot the standard curve by using integral value of peak area as the Y-axis (Y) and mass concentration (mg · ml^{-1}) as the X-axis (X). Meanwhile, signal-to-noise ratio of 3 and signal-to-noise ratio of 10 were selected as the limit of detection

Table 1: Linear relationships of four alkaloid standards

Alkaloid	Regression equation	r^2	Linear range/μg	LOD/ng	LOQ/ng
Arecoline hydrobromide	$Y=20.5\times10^5X+1.64\times10^5$	0.9988	0.0620-4.00	2.60	8.67
Guvacine hydrochloride	$Y=24.5\times10^5X+0.330\times10^5$	0.9990	0.0200-1.30	1.69	5.63
Guvacoline hydrobromide	$Y=25.9\times10^5X+0.228\times10^5$	0.9990	0.0141-0.88	1.17	3.89
Arecaidine hydrochloride	$Y=18.4\times10^5X+0.243\times10^5$	0.9990	0.0201-1.28	1.66	5.53

Table 2: Precision, stability and repeatability (n=6, RSD%)

Standard	Intraday precision	Day-to-day precision	Stability	Repeatability
Arecaidine hydrochloride	0.74	1.28	0.67	0.71
Guvacine hydrochloride	0.74	0.91	0.30	0.24
Arecoline hydrobromide	0.53	0.66	1.36	0.12
Guvacoline hydrobromide	0.59	0.97	0.82	0.57

(LOD) and the limit of quantitation (LOQ) respectively (table 1). The four alkaloids all showed good linear relationships within certain ranges.

Table 3: Recoveries of four alkaloids (n=3).

Standard	Recovery	RSD%
Arecaidine hydrochloride	102.96	1.45
Guvacine hydrochloride	102.26	1.12
Arecoline hydrobromide	96.86	1.82
Guvacoline hydrobromide	102.84	1.75

Precision, stability and repeatability

The mixed standard solution at the same concentration was continuously injected six times to determine the intraday precision. Moreover, the solution was injected twice per day for three consecutive days to determine the day-to-day precision. PA medicinal materials (Guangxi 120613) were prepared into sample solutions and injected at 0, 2, 4, 8, 12 and 24h to examine the stability. In addition, six samples of the same batch were injected to detect the repeatability (table 2). The relative standard deviations of intraday precision, day-to-day precision, stability and repeatability were determined as <0.74%, <1.28%, <1.36% and <0.71% respectively.

Recovery

Nine PA samples (0.25g each) were prepared into sample solutions after adding standards with the alkaloid contents 1.2-, 1.0- and 0.8-fold those in the samples to calculate the recoveries of the four alkaloids (table 3). The recoveries ranged from 96.86% to 102.96%, with RSD<1.82%.

Determination of RCFs

By using arecoline hydrobromide as the internal standard, the RCFs of arecaidine hydrochloride, guvacine hydrochloride and guvacoline hydrobromide at five concentrations were calculated according to Eq. 1 (table 4) as 1.171, 0.879 and 0.806 respectively, with RSD<3.78%. The results met the requirements of QAMS. RCFs did not change obviously when the column temperature, flow rate and proportion of mobile phase were altered, whereas they were evidently affected by the detection wavelength that should thus be optimized.

Positioning of HPLC peaks

Positioning of HPLC peaks is prerequisite for QAMS. For the same system, different HPLC columns gave relative retention value RSD>14.09% and retention time difference RSD>59.58%, which failed to satisfy the requirements of QAMS (table 5). The relative retention times detected by Waters and Thermo columns with different systems are summarized in table 6 and table 7. However, the relative retention values of all peaks had RSD<5% when the same column was used with different systems, which met the requirements of QAMS. Accordingly, SCX columns produced by Thermo Fisher Scientific were chosen by taking resolution and peak time into consideration. The differences between peak position can mainly be attributed to different manufacturers, being associated with the properties and preparation of packing materials. Even the packing material was identical, i.e. strong cation exchange bonded silica particles, the performances of columns from various manufacturers may differ significantly. To this end, we herein restricted conditional parameters to qualify the positioning of HPLC peaks.

Comparison between QAMS and external standard method

The Thermo Fisher Scientific column packed with strong cation exchange bonded silica particles was finally selected considering peak time and symmetry, etc. Under the optimized conditions, the contents of four alkaloids in 12 PA samples were measured by QAMS and external standard method respectively (table 8).

Table 4: RCFs of three alkaloid samples (n=5).

Apparatus	Column	RCF		
		$f_{arecoline/arecaidine}$	$f_{arecoline/guvacine}$	$f_{arecoline/guvacoline}$
Waters	Thermo	1.133	0.847	0.800
	Ultimate	1.124	0.857	0.801
	Waters	1.203	0.902	0.809
Agilent	Thermo	1.179	0.898	0.789
	Waters	1.219	0.893	0.817
Shimadzu	Thermo	1.132	0.860	0.813
	Waters	1.206	0.895	0.810
Mean		1.171	0.879	0.806
RSD (%)		3.78	2.88	1.30

Table 5: Relative retention values and retention time differences detected by different columns with the Waters system (n=5).

Column	Relative retention value			Retention time difference		
	$r_{arecoline/arecaidine}$	$r_{arecoline/arecaidine}$	$r_{arecoline/arecaidine}$	$\Delta t_{arecoline/arecaidine}$	$\Delta t_{arecoline/guvacine}$	$\Delta t_{arecoline/guvacoline}$
Thermo	0.726	0.849	1.080	3.174	1.686	1.119
Ultimate	0.780	0.959	1.321	9.302	22.875	7.573
Waters	0.500	0.537	0.941	38.802	36.184	4.588
Mean	0.677	0.788	1.117	17.093	20.248	4.427
RSD%	18.14	22.87	14.09	90.99	70.16	59.58

Table 6: Relative retention values detected by Waters column with different systems (n=6).

Apparatus	Column	$r_{arecoline/arecaidine}$	$r_{arecoline/guvacine}$	$r_{arecoline/guvacoline}$
Waters	Waters1	0.5	0.537	0.941
	Waters2	0.495	0.522	0.875
	Waters3	0.512	0.515	0.92
Agilent	Waters1	0.452	0.495	0.945
	Waters2	0.481	0.513	0.976
	Waters3	0.476	0.502	0.961
Shimadzu	Waters1	0.469	0.51	0.943
	Waters2	0.473	0.522	0.935
	Waters3	0.461	0.529	0.955
Mean		0.480	0.521	0.944
RSD (%)		4.02	3.21	3.07

Table 7: Relative retention values detected by three Thermo Fisher Scientific columns with different systems (n=5).

Apparatus	Column	Relative retention value		
		$r_{arecoline/arecaidine}$	$r_{arecoline/guvacine}$	$r_{arecoline/guvacoline}$
Waters	Thermo1	0.670	0.800	1.130
	Thermo2	0.726	0.849	1.080
	Thermo3	0.673	0.753	1.069
Agilent	Thermo1	0.697	0.770	1.097
	Thermo2	0.658	0.741	1.078
	Thermo3	0.669	0.747	1.018
Shimadzu	Thermo1	0.711	0.783	1.097
	Thermo2	0.673	0.757	1.064
	Thermo3	0.691	0.760	1.027
Mean		0.685	0.773	1.073
RSD (%)		3.27	4.37	3.25

Thermo1, PN: 73205-254630, LOT: 12948, SN: 10206780; Thermo2, PN: 73205-254630, LOT: 11407, SN: 10020173, Thermo3, PN: 73205-254630, LOT: 11301, SN: 12107512Q3.

Table 8: Contents of four alkaloids in PA samples detected by QAMS and external standard method (mg/g, n=3).

Place of origin and batch No.	Arecoline	Arecaidine		Guvacine		Guvacoline	
		External standard method	QAMS	External standard method	QAMS	External standard method	QAMS
Guangdong120308	3.60	1.89	1.85	1.57	1.53	1.15	1.12
Guangxi120613	3.69	2.21	2.17	1.50	1.46	1.17	1.16
Guangxi120601	3.38	1.55	1.52	1.42	1.49	0.85	0.86
Guangxi120415	3.77	1.88	1.90	1.13	1.18	0.81	0.82
Guangxi120308	3.53	2.06	2.13	1.33	1.40	1.00	1.01
Guangxi111118	3.26	1.88	1.88	1.18	1.21	0.88	0.88
Guangxi111107	2.66	1.84	1.85	1.17	1.21	1.00	0.99
Taiwan120801	2.67	2.86	2.89	1.88	1.91	1.06	1.05
Taiwan120701	2.87	2.16	2.20	1.68	1.70	1.22	1.25
Taiwan120510	1.92	2.53	2.56	1.85	1.88	1.40	1.40
Hainan120109	2.51	1.06	1.12	0.83	0.81	0.52	0.51
Yunnan120706	3.80	2.78	2.85	2.26	2.29	1.41	1.43
Mean	3.14	2.06	2.08	1.48	1.51	1.04	1.04

DISCUSSION

Multi-component determination of Chinese material medica has been bottlenecked by the instability and shortage of makers as well as expensive test costs (Lu *et al.*, 2012). The Chinese Pharmacopoeia (2010 Edition) has applied QAMS to the content determination of alkaloids in *C. Chinensis* by using berberine hydrochloride, which is cheap and easily available, as the marker (State Pharmacopoeia Commission of P.R. China, 2010). Recently, particular attention has been paid to QAMS for TCM studies because this accurate, reliable method plays a key role in controlling quality.

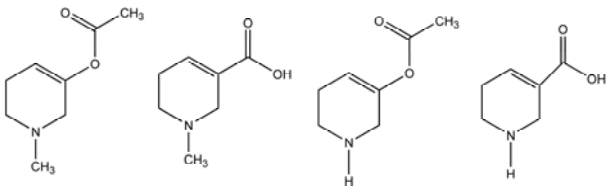

Fig. 1: Structures of main alkaloids in PA. (I) Arecoline; (II) arecaidine; (III) guvacoline; (IV) guvacine.

RCF and HPLC peak positioning predominantly control QAMS. In this study, by employing arecoline as the internal standard, RCFs for arecaidine, guvacine and guvacoline at five concentrations were detected with three HPLC systems and three HPLC columns. The peaks of arecaidine, guvacine and guvacoline were then positioned, during which the columns with the same packing materials from different manufacturers remarkably affected the relative retention values and retention time differences of the alkaloids. Nevertheless, the columns, from different batches, managed to give relative retention values satisfying the requirements of QAMS. Eventually, the Thermo Fisher Scientific column packed with strong cation exchange bonded silica particles was selected by considering resolution and peak time, etc. The RCFs for arecaidine hydrochloride, guvacine hydrochloride and guvacoline hydrobromide were calculated as 1.171, 0.879 and 0.806 respectively. Under the optimized conditions, the contents of four alkaloids in 12 PA samples were measured by QAMS and external standard method respectively for comparison (Chen *et al.*, 2013; Lin *et al.*, 2013).

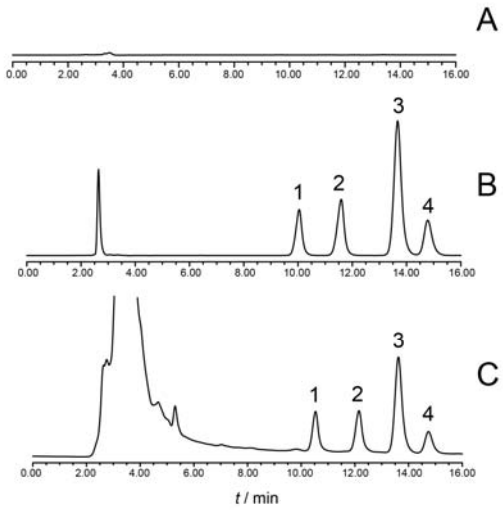

Fig. 2: HPLC results of four alkaloids. A: Solvent; B: mixed standards; C: PA sample. 1: arecaidine; 2: guvacine; 3: arecoline; 4: guvacoline.

When using QAMS in the future, the conditions that affect HPLC peak positioning should be restricted to make this method feasible. The results have never been reported and valuable evidence for economical content determination and quality control of alkaloids in PA. Moreover, the findings also inspire other studies with distinctively different HPLC peak positions.

ACKNOWLEDGMENTS

This study was financially supported by the Standards Improvement Project for Chinese Pharmacopoeia (No. 2011-779), and the Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

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