Regular Article

Identification and Quantification of Alkaloid in KHR98 and Fragmentation Pathways in HPLC-Q-TOF-MS

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Uncaria rhynchophylla is woody climber plant distributed mainly in China and Japan, the stems and hooks of which can be collected as “Gou-Teng” for the treatment of hyperpyrexia, epilepsy and preeclampsia. Fudan University first manufactured KHR98, the extract of *Uncaria rhynchophylla*. In order to study the active components and structural information of KHR98, we established a HPLC coupled with quadrupole time-of-flight (Q-TOF)-MS method for rapid analysis of alkaloids. In qualitative analysis, a total of eight compounds, including four known alkaloids and four unknown components, were detected and identified. The fragmentation behaviors, such as the fragment ion information and the fragmentation pathways of the eight components were summarized simultaneously, and the concentration of the above components was determined by HPLC-MS method. The quantitative method was proved to be reproducible, precise and accurate. This study shed light on the standardization and quality control of the KHR98 and provided a foundation for the further research on pharmacology, follow-up clinical research and New Drug Applications.

Key words *Uncaria rhynchophylla* (UR); alkaloid; fragmentation pathway; identification; qualification; HPLC coupled with quadrupole time-of-flight (Q-TOF)-MS

Uncaria rhynchophylla (UR) is mainly distributed in China and Japan,¹⁾ and is generally used to treat ailments in cardiovascular and central nervous systems, such as dizziness, convulsions, cerebral arteriosclerosis and hypertension, etc.^{2–5)} The phytochemical studies of UR have resulted in the discovery of various types of compounds, including indole alkaloids, triterpenes, flavonoids and phenylpropanoids,⁶⁾ of which the indole alkaloids, for instance, rhynchophylline, isorhynchophylline, corynoxine, isocorynoxine and hirsutine, are commonly recognized as important bioactive ingredients closely related to the pharmacological activities.^{7,8)} These alkaloids exhibit a number of pharmacological effects, such as decreased blood pressure, vasodilatation, sedation and protection against ischemia-induced neuronal damage.^{9,10)} So it is important to separate and analyze those alkaloids. Up till now, multiple researches have been made in this area and several composition analysis methods have been employed. HPLC-UV technique has been adopted in previous researches on alkaloids, such as the study on the screening and identification of six oxindole alkaloids and four indole alkaloids in *Uncaria rhynchophylla* by HPLC.¹¹⁾ Apart from that, the HPLC-MS technique, which is able to provide both analytical separation and structural determination of unknown bioactive compounds, has previously been proposed as a technique of choice for the analysis of these alkaloids from *Uncaria rhynchophylla*^{12,13)}; for instance, ten oxindole alkaloids and four glycosidic indole alkaloids have been identified using LC-atmospheric pressure chemical ionization (APCI)-MS method,¹²⁾ and a total of 29 compounds, comprising 18 alkaloids, 6 flavonoids and 5 quinic acids have been identified employing HPLC-diode array detector (DAD)-quadrupole time-of-flight (Q-TOF)-MS method¹⁾ in previous studies.

KHR98, a dark brown powder, is the extract of a traditional Chinese medicine *Uncaria rhynchophylla*. It was first produced by Fudan University and belonged to the State Category V New Drug. According to the requirements of State Food and Drug Administration (SFDA) for the registration and classification of traditional Chinese medicine and natural medicine,¹⁴⁾ the effective constituents should account for more than 50% of the extracts for the State Category V New Drug. But for KHR98, the alkaloids contained in the effective constituent are not clear. In this study, four kinds of alkaloid components, namely rhynchophylline, isorhynchophylline, corynoxine and isocorynoxine have been detected to contain KHR98. Then, the possible fragmentation pathways of the four kinds of alkaloids were inferred from the accurate molecular weight and fragment information provided by the high resolution mass spectrometer (Q-TOF). Next, the structural information and fragmentation pathways of four unknown components (hirsutine, dihydrocorynantheine, corynantheine, hirsuteine or theirs isomers) were interpreted in KHR98 through higher quality structural information offered by the accurate mass measurement of all the protonated molecules and diagnostic fragment ions. Last, the above components were quantified using HPLC-MS method.

Four known indole alkaloids were detected and four unknown components were identified for KHR98 as a whole. HPLC-MS/MS was used to identify diagnostic fragment ions and interpret fragmentation pathways of known and unknown alkaloids of KHR98. A precise and sensitive HPLC-MS method was validated to simultaneously determine these substances. Therefore this study can be a foundation for further pharmacological study, clinical researches and New Drug Application of this State Category V New Drug, and a reference for future work.

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Table 1. Targeted MS-MS Table

Alkaloid	Prec. m/z	Ret. time (min)	Delta ret. time (min)	Iso. width	Collision energy
Corynoxene	383.1975	15.059	0.5	Medium ($\sim 4m/z$)	25
Isocorynoxene	383.1975	19.175	0.5	Medium ($\sim 4m/z$)	40
Isorhynchophylline	385.2128	14.514	0.5	Medium ($\sim 4m/z$)	25
Rhynchophylline	385.2128	19.370	0.5	Medium ($\sim 4m/z$)	30
Corynantheine	367.2024	17.422	0.5	Medium ($\sim 4m/z$)	30
Hirsutine	367.2024	17.984	0.5	Medium ($\sim 4m/z$)	30
Hirsutine	369.2177	17.431	0.5	Medium ($\sim 4m/z$)	30
Dihydrocorynantheine	369.2177	19.115	0.5	Medium ($\sim 4m/z$)	30

Experimental

Chemicals and Drugs The *Uncaria rhynchophylla* extract, KHR98 (lot number: 43, 44, 45), was kindly provided by Fudan University (FU, Shanghai, China). Standard compounds, including corynoxene, isocorynoxene, rhynchophylline, hirsutine and isorhynchophylline were purchased from Seebio Biotech (Shanghai) Co., Ltd. (Shanghai, China). The purity of all the standard compounds was over 98%. Ammonium acetate, analytical grade, was purchased from Sinpharm Chemical Reagent Co., Ltd. (Shanghai, China). Acetonitrile, HPLC grade, was purchased from Merck (Darmstadt, Germany). Deionized water used for LC-MS/MS and LC-MS was freshly processed through the Milli-Q plus system (Millipore, U.S.A.).

Instrumentation The above alkaloids were carried out by an Agilent 1290 Infinity Series HPLC System (Agilent, Palo Alto, CA, U.S.A.) which was equipped with a pump, an automatic sampler, a column oven and an automatic degasser. A 6538 Accurate-Mass Q-TOF-MS device (Agilent Technologies, CA, U.S.A.) interfaced with an electrospray ionization (ESI) source was employed for the HPLC-MS/MS analyses in positive ionization mode. The raw data acquisition and processing were performed using Analyst software Version 1.7.

HPLC-MS and HPLC-MS/MS Conditions Samples were separated on a Ximate C_{18} column (250×4.6 mm i.d., 5 μ m; Welch®, China). The mobile phase consisted of 10 mM Ammonium acetate solution and acetonitrile at 55:45 (v/v) delivered with a flow rate of 0.8 mL/min. The injection volume was 2 μ L and the column temperature was maintained at 30°C. The divergence ratio was 3:1.

The mass spectrometer was equipped with an ESI source operating in positive ion mode. The following operating parameters were used: drying gas (N_2) flow rate, 12 L/min at 300°C; capillary voltage, 3500 V; atomized pressure, 45 psig; fragmentor voltage, 175 V; Skimmer voltage, 65 V; OCR 1 RF Vpp 750 V; mass range, m/z 100–1000. The exact molecular weight was measured using a ten point correction method (m/z 112.9856, 301.9981, 601.9790, 1033.9881, 1333.9689, 1633.9498, 1933.9306, 2233.9115, 2533.8923, 2833.8731). Other mass parameters are shown in Table 1.

Preparation of Standard and Sample Solutions for Qualitative Analysis

Standard Solutions

Stock solutions (500 μ g/mL) of four standard substances (corynoxene, isocorynoxene, rhynchophylline and isorhynchophylline) were prepared in methanol. Each stock solutions were transferred to a volumetric flask and mixed to make a standard solution with the concentration of 5 μ g/mL.

Fig. 1. Structures of (A) Rhynchophylline, (B) Isorhynchophylline, (C) Corynoxene and (D) Isocorynoxene

Sample Solutions

The stock solutions of KHR98 was 1 mg/mL in methanol. The KHR98 sample solutions were prepared by diluting the KHR98 stock solutions with methanol to the concentration of 50 μ g/mL.

Preparation of Standard and Sample Solutions for Quantitative Analysis

Standard Solutions

Samples for five standard substances (corynoxene, isocorynoxene, rhynchophylline, hirsutine and isorhynchophylline) were accurately weighed and dissolved in methanol to make mixed stock solutions at 10 μ g/mL. The standard solutions were prepared with the appropriate dilution of stock solutions with methanol.

Sample Solutions

This method was in accord with the method of making sample solutions for qualitative analysis.

Linearity

The standard curves were established by plotting peak area ratio of alkaloid over concentration, ranging from 1 to 10 μ g/mL (x-axis). The stock solutions containing corynoxene, isocorynoxene, rhynchophylline, isorhynchophylline and hirsutine were diluted to concentrations of 1, 3, 5, 7 and 10 μ g/mL.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The stock solutions containing five standard substances were diluted to concentrations ranging from 0.01 μ g/mL to 1 μ g/mL. Under the present chromatographic conditions, the limits of LOD and LOQ were determined at a signal-to-noise ratio (S/N) of 3 and 10, respectively.

Precision

Took the standard solutions with the concentrations of 1, 5 and 10 μ g/mL to analysis under corresponding chromato-

Fig. 2. TIC Chromatography

Fig. 3. MS/MS Spectra of (A) Rhynchophylline, (B) Isorhynchophylline, (C) Corynoxine and (D) Isocorynoxine

Fig. 4. (1) The Proposed Fragmentation Pathways of Rhynchophylline and Isorhynchophylline; (2) The Proposed Fragmentation Pathways of Corynoxine and Isocorynoxine

graphic conditions.

Recovery

Took the standard solutions with the concentrations of 3, 5 and 7 $\mu\text{g/mL}$ to analysis under corresponding chromatographic conditions.

Results and Discussion

The Determination of the Known Alkaloids in KHR98

We analyzed the accurate molecular weight provided by Q-TOF-MS and the retention time from chromatography, compared the information with the standard substances, and then detected rhynchophylline, isorhynchophylline, corynoxine and isocorynoxine in KHR98 (Fig. 1). The total ion chromatogram (TIC) chromatography was shown in Fig. 2.

MS/MS Fragment Pathways of Rhynchophylline and Isorhynchophylline

According to the accurate molecular weight and fragmentation information provided by HPLC-Q-TOF-MS, it was likely to deduce the possible fragmentation pathways of compounds.

In positive MS mode, some fragmentation information could be delineated on the basis of the mass fragmentation pathways and the intensities of the ion peaks.¹¹⁾ As shown in Figs. 3(A) and 3(B), rhynchophylline and isorhynchophylline displayed an accurate quasi-molecular ion $[M+H]^+$ at m/z 385.2122, the molecular formula was C₂₂H₂₉N₂O₄⁺, and the characteristic fragmentation ions appeared at m/z 226.14,

353.19, 213.10, 269.16, 187.09 and 160.08. The possible fragmentation pathways of rhynchophylline and isorhynchophylline could thus be inferred and the fragments observed further proved our prediction of those compositions (Fig. 4(1)). The loss of C₁₀H₉NO (159 Da) and methoxyl moiety (32 Da) unit from rhynchophylline and isorhynchophylline led to the emergence of the ion **1b** at m/z 226.14 and the ion **1c** at m/z 353.19. The fragment ion **1d** (m/z 269.16) could be explained by the elimination of C₄H₄O₂ (84 Da) on **1c**. Losing C₄H₈ (56 Da) and C₇H₁₁N (109 Da), **1d**, as shown in its MS/MS spectrum, gave rise to the major fragment ions **1f** (m/z 160.08) and **1e** (m/z 213.10) which in turn generated ion **1g** (m/z 187.09) with the removal of C₂H₂ (28 Da) segment from **1e**.

MS/MS Fragment Pathways of Corynoxine and Isocorynoxine

As shown in Figs. 3(C) and 3(D), the Q-TOF mass spectrometry of corynoxine and isocorynoxine saw the $[M+H]^+$ ion at m/z 383.1965, corresponding to the molecular formula C₂₂H₂₇N₂O₄⁺. A series of ions (m/z 215.12, 351.17, 213.10, 267.15, 187.09, 160.08 and 172.08) came out when corynoxine and isocorynoxine served as the parent ions. The possible fragmentation pathways of corynoxine and isocorynoxine were deduced and shown in Fig. 4(2). The appearance of the ion peaks at m/z 215.12 (**2b**) and m/z 351.17 (**2c**) could be attributed to the loss of C₉H₁₂O₃ (168 Da) and methoxyl moiety (32 Da) unit from **2a**. **2d** at m/z 213.10, which was created by

Fig. 5. E(1)-1 MS/MS Spectra of the Unknown Alkaloids (t_R = 17.431 min); E(1)-2 MS/MS Spectra of the Unknown Alkaloids (t_R = 19.115 min); E(2) The Proposed Fragmentation Pathways of Alkaloids A1 and A2; E(3) Chemical Structures of Alkaloids A1 and A2 Identified in KHR98

2b after the elimination of two hydrogen atoms (2Da), could thus form ion **2e** (m/z 187.09) after the removal of the chemical group C_7H_2 (26Da). Then **2f** (m/z 172.08) was produced with **2e** losing NH (15Da). As for another pathway, **2h** (m/z 160.08) was shaped after stripping C_7H_9N group (107Da) from **2g** (m/z 267.15), which had the same structure as **2c** after adding a chemical unit of $C_4H_4O_2$ (84Da).

The Identification of the Unknown Alkaloids in KHR98 Four main unknown alkaloids were identified in KHR98, their $[M+H]^+$ ions were used as the precursor ions to optimize the collision energy and the MS/MS spectra were as well collected. Based on the fragmented ion information, the fragmentation regularity and relevant researches, the possible structure of the unknown components was deduced.

Structural Characterization and MS/MS Fragment Pathways of Alkaloids A1 and A2 Two unknown alkaloids (called alkaloids A1 and A2) were identified in KHR98, with their parent ion $[M+H]^+$ being m/z 369.2173 and the retention time at 17.431 and 19.115 min, respectively. The MS/MS spectra were shown in Figs. 5E(1)-1 and 5E(1)-2. Ions at m/z 337.19, 251.15, 238.14, 226.14 and 170.10 could be used as characteristic fragmentation ions for alkaloids A1 and A2. According to the literature researches, alkaloids A1 and A2 may

Fig. 5. F(1)-1 MS/MS Spectra of the Unknown Alkaloids (t_R = 17.422 min); F(1)-2 MS/MS Spectra of the Unknown Alkaloids (t_R = 17.894 min); F(2) The Proposed Fragmentation Pathways of Alkaloids B1 and B2; F(3) Chemical Structures of Alkaloids B1 and B2 Identified in KHR98

be hirsutine, dihydrocorynantheine or their isomers, whose structures were shown in Fig. 5E(3).¹⁵⁻¹⁸ The possible fragment pathways were shown in Fig. 5E(2). In positive mode, the analyses of alkaloids A1 and A2 indicated the $[M+H]^+$ ion (**3a**) was at m/z 369.2173, corresponding to the molecular formula $C_{22}H_{29}N_2O_3^+$. Compounds **3a** produced **3b**, **3c**, **3d** and **3e**, suggesting the loss of $C_5H_9O_3$ (118Da), $C_{10}H_9N$ (143Da), C_9H_9N (131Da) and methoxyl moiety (32Da). The fragment ion **3b** generated the ion **3f** at m/z 170.10 due to the loss of C_5H_9N (81Da).

Structural Characterization and MS/MS Fragment Pathways of Alkaloids B1 and B2 Other two unknown alkaloids (called alkaloids B1 and B2) were identified in the KHR98, with their parent ion $[M+H]^+$ being m/z 367.2024 and the retention time at 17.422 and 17.894 min, respectively. The MS/MS spectra were shown in Figs. 5F(1)-1 and 5F(1)-2, in which a series of ions at m/z 335.18, 170.10, 238.14, 251.15 and 224.13 arose from the alkaloids B1 and B2 as characteristic fragmentation ions. Based on literature data, alkaloids B1 and B2 were assigned as corynantheine, hirsutine or their isomers, the structures of which were shown in Fig. 5F(3).^{15,16,19} and the possible fragment pathways of which were

Fig. 6. EIC of Reference Solution (A. EIC=383.2, 1: Corynoxene, 2: Isocorynoxene; B. EIC=385.2, 3: Isorhynchophylline, 4: Rhynchophylline; C. EIC=369.2, 5: Hirsutine)

Fig. 7. EIC of Sample Solution (A. EIC=383.2, 1: Corynoxene, 2: Isocorynoxene; B. EIC=385.2, 3: Isorhynchophylline, 4: Rhynchophylline; C. EIC=369.2, 5: Hirsutine, 6: Alkaloid A2; D. EIC=367.2, 7: Alkaloid B1, 8: Alkaloid B2)

Table 2. Linear Regression, LOD and LOQ of Five Investigated Compounds

Alkaloid	Linearity range ($\mu\text{g/mL}$)	Regression equation ($n=5$)	r^2	LOD ($\mu\text{g/mL}$)	LOQ ($\mu\text{g/mL}$)
Rhynchophylline	1.16–11.6	$y=2576251x+178847$	0.9994	0.04	0.22
Isorhynchophylline	1.09–10.9	$y=2461471x+114735$	0.9993	0.03	0.18
Corynoxene	1.3–13.0	$y=3636004x+642168$	0.9991	0.08	0.58
Isocorynoxene	1.2–12.0	$y=2705735x+198143$	0.9996	0.07	0.45
Hirsutine	1.02–10.2	$y=2489374x+209347$	0.9995	0.03	0.19

Table 3. Inter-day and Intra-day of Five Investigated Compounds

Alkaloid	Intra-day precisions RSD (%)			Inter-day precisions RSD (%)		
	Low	Medium	High	Low	Medium	High
Rhynchophylline	1.46	1.39	1.52	1.74	1.82	1.99
Isorhynchophylline	1.83	1.57	1.31	2.35	1.92	2.28
Corynoxene	1.36	1.44	1.57	1.84	1.63	2.17
Isocorynoxene	1.32	1.09	1.83	1.32	1.83	2.31
Hirsutine	1.74	1.59	1.37	1.84	1.07	1.75

Table 4. Recovery of Five Investigated Compounds

Alkaloid	Recovery (%)		
	Low	Medium	High
Rhynchophylline	98.39	100.86	100.36
Isorhynchophylline	97.57	99.28	99.11
Corynoxine	98.45	99.39	101.12
Isocorynoxine	98.35	101.02	100.62
Hirsutine	97.83	100.83	101.26

Table 5. Contents of Eight Major Compounds in Three Batches of KHR98

Batch number	Content (mg/g)		
	43	44	45
Rhynchophylline	53.9	69.1	58.3
Isorhynchophylline	86.9	83.5	101.4
Corynoxine	79.3	67.0	64.9
Isocorynoxine	93.2	106.9	91.7
Hirsutine	31.0	54.0	45.0
Dihydrocorynantheine	3.0	5.0	6.0
Corynantheine	40.0	64.0	39.0
Hirsuteine	38.9	53.0	53.0
Total content	426.2	502.5	459.3

shown in Fig. 5F(2). Compound **4a** produced ions **4b–4d** in which **4b** (m/z 335.18), **4c** (m/z 170.10) and **4d** (m/z 238.14) were assigned to the elimination of methoxyl moiety (32 Da), $C_{10}H_{15}NO_3$ (197 Da), and C_9H_7N (129 Da). Then **4b** lost $C_4H_4O_2$ (84 Da) to generate ions **4e** (m/z 251.15). The ion **4f** (m/z 224.13) arose from **4d** losing CH_2 (14 Da).

Method Validation of the HPLC-MS Analysis

In this study, five marker compounds, namely corynoxine, isocorynoxine, rhynchophylline, isorhynchophylline and hirsutine, were simultaneously quantified by the developed quantitative method. SFDA guidelines were the foundation for establishing this analytical method. Extracted ion chromatography (EIC) mode with a four-section switch was employed for quantification. The corynoxine and isocorynoxine were at m/z 383.2, rhynchophylline and isorhynchophylline were at m/z 385.2, hirsutine and dihydrocorynantheine were at m/z 369.2, corynantheine and hirsuteine were at m/z 367.2. The typical chromatograms for a standard solution and sample solution were shown in Figs. 6 and 7, which clearly indicated good separation could be achieved. Tables 2–4 demonstrated the results of method validation. Good linear relationships for all alkaloid were shown ($r^2 \geq 0.9991$) and the LODs and LOQs were discovered to be in the ranges from 0.03–0.08 $\mu\text{g/mL}$ to 0.18–0.58 $\mu\text{g/mL}$ (Table 2). For all five compounds, the Relative Standard Deviation (RSD) of intraday and interday precision were found to be within the scope of 1.09–1.83 and 1.07–2.35% (Table 3). The average recoveries were calculated and the observed alkaloids exhibited good recovery rates of 97.57–101.26% (Table 4). The five alkaloids (corynoxine, isocorynoxine, rhynchophylline, isorhynchophylline and hirsutine) turned out to be stable in the sample solution in 12h at indoor temperature, with RSD values at 1.96, 1.51, 2.05, 2.42 and 2.73%.

Alkaloids consisted in KHR98 were investigated under the above chromatographic conditions and by the quantification method proposed. According to the qualitative results of the unknown compositions of KHR98 by HPLC-Q-TOF-MS, a new reference standard, hirsutine (alkaloid A1), was purchased. For those alkaloid components of existing standard (rhynchophylline, isorhynchophylline, corynoxine, isocorynoxine and hirsutine), the external standard method was used. For other alkaloids, dihydrocorynantheine (alkaloid A2), corynantheine (alkaloid B1), hirsuteine (alkaloid B2), Their reference standards were not easy to obtain, the content of each alkaloid component was calculated based on the similar structure of hirsutine. On the one hand, dihydrocorynantheine, corynantheine, hirsuteine and hirsutine have a similar structure, dihydrocorynantheine and hirsutine, corynantheine and hirsuteine are geometric isomers, hirsutine and dihydrocorynantheine have more than two hydrogen atoms than corynantheine and hirsuteine. On the other hand, these four substances have resemble fragmentation pattern, for instance, α -cleavage, DRA-cleavage, i-cleavage and so on, and we determined the position of the peaks by the results of HPLC-Q-TOF-MS. Eight compounds were quantified simultaneously and the results were displayed in Table 5.

Conclusion

To summarize, the possible fragmentation pathway of the four kinds of alkaloids components in KHR98, namely rhynchophylline, isorhynchophylline, corynoxine and isocorynoxine have been inferred with the proposed HPLC-Q-TOF-MS method. In addition, the main unknown components of KHR98 have been investigated, and the structures and possible fragmentation pathways have been founded and summarized. At last, eight marker compounds have been simultaneously quantified and their contents have been determined with the proposed HPLC-MS method. These pre-clinical data provide the first firm basis for the clinical researches, and our data can be used for New Drug Application and quality assessment of KHR98.

Conflict of Interest The authors declare no conflict of interest.

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