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, and is generally used to treat ailments in cardiovascular and central nervous systems, such as dizziness, convulsions, cerebral arteriosclerosis and hypertension, etc. The phytochemical studies of UR have resulted in the discovery of various types of compounds, including indole alkaloids, triterpenes, flavonoids and phenylpropanoids, of which the indole alkaloids, for instance, rhynchophylline, isocorynoxeine and hirsutine, are commonly recognized as important bioactive ingredients closely related to the pharmacological activities. These alkaloids exhibit a number of pharmacological effects, such as decreased blood pressure, vasodilatation, sedation and protection against ischemia-induced neuronal damage. So it is important to separate and analyze those alkaloids. Until 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. 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. For instance, ten oxindole alkaloids and four glycosidic indole alkaloids have been identified using LC-atmospheric pressure chemical ionization (APCI)-MS method 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 method 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, 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, corynoline and isocorynoline 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 compounds (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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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 corynxeine, isocorynxeine, rhynchophylline, hirsutine andisorhynchophylline 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 ion 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 Xtimate C18 column (250×4.6 mm i.d., 5 μm; 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 μL 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 (N2) 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 μg/mL) of four standard substances (corynxeine, isocorynxeine, 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 μg/mL.

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 μg/mL.

Preparation of Standard and Sample Solutions for Quantitative Analysis

Standard Solutions  Samples for five standard substances (corynxeine, isocorynxeine, rhynchophylline, hirsutine andisorhynchophylline) were accurately weighed and dissolved in methanol to make mixed stock solutions at 10 μg/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 μg/mL (x-axis). The stock solutions containing corynxeine, isocorynxeine, rhynchophylline, isorhynchophylline and hirsutine were diluted to concentrations of 1, 3, 5, 7 and 10 μg/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 μg/mL to 1 μg/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 μg/mL to analysis under corresponding chromatogram.

Table 1. Targeted MS-MS Table

| Alkaloid               | Prec. m/z | Ret. time (min) | Delta ret. time (min) | Iso. width | Collision energy |
|------------------------|-----------|-----------------|------------------------|------------|------------------|
| Corynxeine             | 383.1975  | 15.059          | 0.5                    | Medium     | 25               |
| Isocorynxeine          | 383.1975  | 19.175          | 0.5                    | Medium     | 40               |
| Isorhynchophylline     | 385.2128  | 14.514          | 0.5                    | Medium     | 25               |
| Rhynchophylline        | 385.2128  | 19.370          | 0.5                    | Medium     | 30               |
| Corynantheine          | 367.2024  | 17.422          | 0.5                    | Medium     | 30               |
| Hirsutine              | 367.2024  | 17.984          | 0.5                    | Medium     | 30               |
| Hirsutine              | 369.2177  | 17.431          | 0.5                    | Medium     | 30               |
| Dihydrocorynantheine   | 369.2177  | 19.115          | 0.5                    | Medium     | 30               |

Fig. 1. Structures of (A) Rhynchophylline, (B) Isorhynchophylline, (C) Corynxeine and (D) Isocorynxeine
Fig. 2. TIC Chromatography

Fig. 3. MS/MS Spectra of (A) Rhynchophylline, (B) Isorrhynchophylline, (C) Corynorchline and (D) Isocorynorchline
graphic conditions.

Recovery
Took the standard solutions with the concentrations of 3, 5 and 7 µg/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, corynoxeine and isocorynoxeine 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. 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 C_{22}H_{29}N_{2}O_{4}+, 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 C_{10}H_{9}NO (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 C_{4}H_{4}O (84 Da) on 1c. Losing C_{5}H_{8} (56 Da) and C_{7}H_{11}N (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 C_{2}H_{2} (28 Da) segment from 1e.

MS/MS Fragment Pathways of Corynoxeine and Isocorynoxeine
As shown in Figs. 3(C) and 3(D), the Q-TOF mass spectrometry of corynoxeine and isocorynoxeine saw the [M+H]+ ion at m/z 383.1965, corresponding to the molecular formula C_{22}H_{27}N_{2}O_{4}+. A series of ions (m/z 215.12, 351.17, 213.10, 267.15, 187.09, 160.08 and 172.08) came out when corynoxeine and isocorynoxeine served as the parent ions. The possible fragmentation pathways of corynoxeine and isocorynoxeine 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 (2e) could be attributed to the loss of C_{6}H_{13}O_{3} (168 Da) and methoxyl moiety (32 Da) unit from 2a. 2d at m/z 213.10, which was created by...
after the elimination of two hydrogen atoms (2 Da), could thus form ion \(2e\) (m/z 187.09) after the removal of the chemical group C\(_2\)H\(_2\) (26 Da). Then \(2f\) (m/z 172.08) was produced with \(2e\) losing NH (15 Da). As for another pathway, \(2h\) (m/z 160.08) was shaped after stripping C\(_7\)H\(_9\)N group (107 Da) from \(2g\) (m/z 267.15), which had the same structure as \(2c\) after adding a chemical unit of C\(_4\)H\(_4\)O\(_2\) (84 Da).

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 [M+H]\(^+\) ions 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 be hirsutine, dihydrocorynantheine or theirs isomers, whose structures were shown in Fig. 5E(3). The possible fragmentation 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\(_2\)O\(_3\). Compounds 3a produced 3b, 3c, 3d and 3e, suggesting the loss of C\(_5\)H\(_{10}\)O\(_3\) (118 Da), C\(_{10}\)H\(_9\)N (143 Da), C\(_9\)H\(_9\)N (131 Da) and methoxyl moiety (32 Da). The fragment ion 3b generated the ion 3f at m/z 170.10 due to the loss of C\(_7\)H\(_7\)N (81 Da).

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, hirsuteine or theirs isomers, the structures of which were shown in Fig. 5F(3). and the possible fragment pathways of which were
Fig. 6. EIC of Reference Solution (A. EIC=383.2, 1: Corynoxeine, 2: Isocorynoxeine; 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: Corynoxeine, 2: Isocorynoxeine; 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 (µg/mL) | Regression equation (n=5) | $r^2$ | LOD (µg/mL) | LOQ (µg/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        |
| Corynoxeine        | 1.3–13.0                | $y=3636004x+642168$        | 0.9991| 0.08        | 0.58        |
| Isocorynoxeine     | 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  |
| Corynoxeine        | 1.36 | 1.44   | 1.57 | 1.84 | 1.63   | 2.17  |
| Isocorynoxeine     | 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  |
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, corynoxeine, isocorynoxeine 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, hirsute and dihydrocorynantheine have more than two hydrogen atoms than corynantheine and hirsutine. On the other hand, these four substances have resemble fragmentation pattern, for instance, α-cleavage, DRA-cleavage, β-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, corynoxeine and isocorynoxeine 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.

### References and Notes

1. Xie S., Shi Y., Wang Y., Wu C., Liu W., Feng F., Xie N. J. Pharm. Biomed. Anal., 81–82, 56–64 (2013).
2. Geng C. A., Huang X. Y., Ma L. B., Hou B., Li T. Z., Zhang X. M., Chen J. J. Nat. Prod., 80, 959–964 (2017).
3. Shi J. S., Yu J. X., Chen X. T., Xu R. X., Acta Pharmacol. Sin., 24, 97–101 (2003).
4. Jiang W. W., Su J., Wu X. D., He J., Peng L. Y., Cheng X., Zhao Q. J. Pharm. Biomed. Anal., 81–82, 56–64 (2013).
5. Lee J. S., Kim J., Kim B. Y., Lee H. S., Ahn J. S., Chang Y. S., J. Nat. Prod. Res., 29, 842–847 (2015).
6. Heitzman M. E., Neto C. C., Winiarz E., Vaisberg A. J., Hammond G. B., Phytochemistry, 66, 5–29 (2005).
7. Zhang Y. B., Yang W. Z., Yao C. L., Feng R. H., Yang M., Guo D. A., Wu W. Y., Fitoterapia, 96, 39–47 (2014).
8. Hui S., Yang Y., Mi Z., Zhu G. X., Qi A., Ji W., Zhu Z., Neuroscience, 337, 355–369 (2016).
9. Yuan D., Ma B., Wu C., Yang J., Zhang L., Liu S., Wu L., Kano Y., J. Nat. Prod., 71, 1271–1274 (2008).
10. Kong F., Ma Q., Huang S., Yang S., Fu L., Zhou L., Dai H., Yu Z., J. Pharm. Biomed. Anal., 81–82, 56–64 (2013).
Zhao Y., Nat. Prod. Res., 31, 1403–1408 (2017).
11) Wang H. B., Qi W., Zhang L., Yuan D., Chem. Pharm. Bull., 62, 1100–1109 (2014).
12) Ou J., Gong T., Ma B., Zhang L., Kano Y., Yuan D., Chem. Pharm. Bull., 60, 23–30 (2012).
13) Montoro P., Carbone V., Quiroz Jde D., De Simone F., Pizza C., Phytochem. Anal., 15, 55–64 (2004).
14) State Food and Drug Administration Bureau of Traditional Chinese Medicine, “Natural Drug Registration Classification and Declaration of Information Requirements [EB/OL]”: http://www.sda.gov.cn/WS01/CL0053/24529_9.html, 2007.
15) Phillipson J. D., Supavita N., Phytochemistry, 22, 1809–1813 (1983).
16) Phillipson J. D., Hemingway S. R., Ridsdale C. E., Part V, Lloydia, 41, 503–570 (1978).
17) Laus G., Brössner D., Keplinger K., Phytochemistry, 45, 855–860 (1997).
18) Ponglux D., Supavita T., Verpoorte R., Phillipson D., J. Pharm. Pharmacol., 32 (S1), 2013–2016 (1980).
19) Laus G., Teppner H., Phyton, 36, 185–196 (1996).