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Short communication

Quality assessment of Rhizoma et Radix Notopterygii by HPTLC and HPLC fingerprinting and HPLC quantitative analysis

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Abstract

This paper describes an improved quality assessment method for Rhizoma et Radix Notopterygii (the rhizome and root of *Notopterygium incisum* Ting ex H.T. Chang or *Notopterygium forbesii* Boiss). The method was established by using fingerprinting and quantitation of marker compounds (isoimperatorin, notopterol and bergapten) in this herbal medicine. The authentication of Rhizoma et Radix Notopterygii using high performance thin-layer chromatography (HPTLC) fingerprinting was achieved by comparing the colors and R_f values of the bands in TLC fingerprints with those of the marker compounds. The HPLC fingerprints of 16 batches of herbal samples from different regions of China showed similar chromatographic patterns. Five peaks were selected as characteristic peaks, and three of these were identified by using LC–MS–MS techniques. The relative retention times of these characteristic peaks in the HPLC fingerprint were established as an important parameter for identification of Rhizoma et Radix Notopterygii. Finally, the pharmacologically active marker compounds isoimperatorin, notopterol and bergapten in this herb were quantitatively determined using a validated reverse-phase HPLC method.

Keywords: *Notopterygium incisum*; *Notopterygium forbesii*; HPLC; Fingerprinting; Isoimperatorin; Notopterol; Bergapten

1. Introduction

Rhizoma et Radix Notopterygii (Qianghuo), the dried rhizome and root of *Notopterygium incisum* Ting ex H.T. Chang or *Notopterygium forbesii* Boiss (family Umbelliferae), is a well-known traditional Chinese medicine that is used widely as an anti-rheumatic and pain-relieving herb for the treatment of colds and rheumatism [1]. Previous research on the chemical constituents of Rhizoma et Radix Notopterygii has shown that it contains a variety of coumarins, sugars, amino acids, organic acids, mono and sesquiterpenes, polyacetylenes and phenolic compounds [2–5]. Pharmacological studies have indicated that coumarins such as isoimperatorin, notopterol and bergapten possess anti-inflammatory, analgesic, anti-cancer and anti-coagulant activities [6–8].

Because the clinical use of Rhizoma et Radix Notopterygii and its medicinal products is common in China, a reasonable assessment method for evaluating the quality of this crude drug is essential to ensure the efficiency and stability of its products. In Chinese Pharmacopoeia (2005 edition), the quantitation of total essential oils was set as the parameter for quality control of this herb [1]. Authentication of Rhizoma et Radix Notopterygii using chromatographic fingerprinting techniques such as TLC and HPLC have been rarely reported. Most of these previous reports focused on the quantitative analysis of one or two constituents using TLC and HPLC [9–12]. Recently, an HPLC fingerprinting study of the water extract of "Qianghuo" was conducted, and ferulic acid, imperatorin and isoimperatorin were identified [13]. To establish an improved quality assessment method for Rhizoma et Radix Notopterygii, in the present paper high performance thin-layer chromatography (HPTLC) fingerprinting and HPLC fingerprinting of multiple components in this herb were conducted. These fingerprinting chromatograms can provide sufficient qualitative information

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for the identification and authentication of Rhizoma et Radix Notopterygii. Sixteen batches of authenticated herbal drugs, originating from two species of the genus *Notopterygium*, were collected from different regions of China for the analysis. In addition, three pharmacologically active components (isoimperatorin, notopterol and bergapten) in the herbal medicine were quantitatively determined by using reverse-phase HPLC for the purpose of quality assessment.

2. Experimental

2.1. Herbal materials

Ten and six samples of Rhizoma et Radix Notopterygii derived from *N. incisum* and *N. forbesii*, respectively, were collected from different regions of China and were air-dried according to the procedure described in Chinese Pharmacopoeia (2005 edition). All of the samples were identified by one of the authors (Z. Zhao) and deposited in the Centre of Chinese Materia Medica, Hong Kong Baptist University.

2.2. Standards and reagents

Isoimperatorin and bergapten (purity > 98%) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and from Aldrich, respectively. Notopterol (purity > 98%) was isolated in our laboratory, and its identity and purity were confirmed by chromatographic methods (TLC and HPLC) and by comparison of ^{1}H NMR and MS data with the published data [14,15].

HPLC-grade acetonitrile and analytical-grade methanol, toluene and ethyl acetate were purchased from Merck (Darmstadt, Germany). Water for HPLC analysis was prepared using a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA).

2.3. HPTLC procedure for identification of herbal samples

One gram powdered herbal sample was extracted with 10 mL methanol for 30 min by sonication. After centrifugation, the supernatant was filtered through a 0.45 μm RC filter for TLC analysis. HPTLC silica gel 60 F_{254} plates (10 cm × 10 cm, Merck) were heated for 60 min at 55 °C before use. Test solutions (1 μL) of the reagent blank, sample, sample duplicate, reference standard and spiked sample (1 μL of sample + 1 μL of isoimperatorin + 1 μL of notopterol) were applied to the plates with a TLC system (Camag, Muttenz, Switzerland). The plates were then developed in a double-trough, 10 cm × 10 cm chamber with a stainless steel lid. The chamber was pre-equilibrated with the mobile phase (toluene and ethyl acetate, (4:1, v/v)). After developing over a path of 8 cm, the plate was air-dried, and the image was captured under UV light (366 nm).

2.4. HPLC fingerprinting

The powdered herbal sample (0.2 g) was extracted with 20 mL methanol for 60 min by sonication. After centrifugation, the supernatant was filtered through a 0.45 μm RC filter into an HPLC amber sample vial for HPLC fingerprinting analysis.

An HP 1100 system (Hewlett-Packard, Wilmington, DE, USA) consisting of a G1312A binary pump, G1329A automatic sample injector and G1315A diode array detector was used to perform HPLC analysis. The HPLC fingerprinting was carried out on a C_{18} column (Alltech, Alltima C_{18}, 4.6 mm × 250 mm, 5 μm) at ambient temperature with a sample injection volume of 10 μL. The mobile phase consisted of water (A) and acetonitrile (B) using a gradient program of 40% B for 0–5 min, 40–70% B for 5–45 min and 70–100% B for 45–50 min. The flow rate was 0.8 mL/min. The fingerprint chromatograms were recorded at an optimized wavelength of 255 nm. The peaks in HPLC fingerprints were identified by comparing the retention times in the chromatograms of extracts with those of authentic reference standards and were confirmed by using LC–MS–MS.

LC–MS–MS analysis was carried out by using a C_{18} column (Alltech, Alltima C_{18}, 2.1 mm × 250 mm, 5 μm) at ambient temperature with a sample injection volume of 5 μL. The mobile phase and gradient program were identical to those used in HPLC–DAD for HPLC fingerprinting, except the flow rate was set at 0.2 mL/min. A Perkin-Elmer Sciex API 365 triple-quadrupole mass spectrometer (Sciex, Toronto, Canada) equipped with an ion-spray (pneumatically assisted electrospray) interface was employed.

2.5. HPLC quantitation of isoimperatorin, notopterol and bergapten

Standard stock solutions of isoimperatorin, notopterol and bergapten, respectively, were prepared by dissolving each compound in methanol to obtain a concentration of 1000 mg/L for isoimperatorin and notopterol and 100 mg/L for bergapten. The concentrations of isoimperatorin, notopterol and bergapten reference standards used for calibration were 5, 20, 60, 100 and 200 mg/L; 1, 10, 50, 100 and 200 mg/L; and 0.5, 1, 5, 10 and 20 mg/L in methanol, respectively.

2.5.1. Sample preparation for HPLC quantitation

The powdered herbal sample (0.2 g) was extracted with 20 mL methanol for 60 min by sonication. After centrifugation, the supernatant was filtered through a 0.45 μm RC filter into an HPLC amber sample vial for HPLC quantitative analysis.

2.5.2. Conditions for HPLC quantitation

The HPLC analysis was carried out on a C_{18} column (Alltech, Alltima C_{18}, 4.6 mm × 250 mm, 5 μm) at ambient temperature with a sample injection volume of 10 μL. The mobile phase consisted of water (A) and acetonitrile (B), using a gradient program of 50–70% B for 0–30 min. The flow rate was 0.8 mL/min. The optimized detection wavelength was 255 nm.

2.5.3. Validation of quantitative method

The extraction efficacy was investigated by comparing the results of the same batch of sample with different sonication times, using methanol as the extraction solvent. A five-point calibration curve was used for linear regression analysis. The

intraday precision was calculated by analysing the midpoint standard solutions of the calibration curve five times. Five replicate analyses of one batch of Rhizoma et Radix Notopterygii sample were carried out to evaluate the repeatability of the quantitative procedure. The recovery studies were conducted by spiking isoimperatorin at ~2.5 mg/g, notopterol at ~5.0 mg/g and bergapten at ~0.25 mg/g, respectively, to the same batch of Rhizoma et Radix Notopterygii sample used in the repeatability study. The limit of detection and limit of quantification were determined by visual evaluation (signal-to-noise ratio of 3:1 and 10:1, respectively).

3. Results and discussion

3.1. HPTLC fingerprinting identification

The performance of TLC using a sorbent with a homogeneous particle size of ~5 μm and with a narrow particle size distribution has been confirmed to be superior to that of the conventional TLC plate that was used in many pharmacopoeias for the identification of herbal materials. Therefore, in this study HPTLC plates were used to establish a TLC fingerprinting method. The chromatographic conditions, in particular the developing solvents (i.e., types of solvents and ratio), were carefully optimized before the 16 batches of crude drug samples were analysed. The results observed under UV light showed a good separation for all compounds in Rhizoma et Radix Notopterygii. Reference marker compounds of isoimperatorin and notopterol were separated on the same plates for the authentication of each herb sample. The samples were identified by matching the colors and R_f values of bands in their fingerprints with those of marker compounds. For positive identification, the sample must exhibit bands with chromatographic characteristics, including colors and R_f values (~0.61 for isoimperatorin and ~0.18 for notopterol), similar to those of reference marker compounds (Fig. 1).

3.2. Optimization of HPLC conditions for fingerprinting and quantitation

The choice of detection wavelength is crucial for developing a reliable fingerprint and for accurate quantitative analysis of marker compounds in the herb. The optimal detection wavelength in the HPLC analysis was determined to be 255 nm. At this wavelength, more characteristic peaks in the fingerprinting chromatogram were observed, and the marker compounds were sensitively detected in the HPLC quantitation. The HPLC separation conditions, such as choices of mobile phase and gradient program, were further optimized. A number of mobile phases with different gradients were screened in order to obtain a reliable chromatogram with most peaks at acceptable resolution and balance for the HPLC fingerprinting and to obtain baseline separation of each marker compound (resolution factor > 1.5) in a relatively short analytical time for the HPLC quantitation. Finally, two gradient programs with water and acetonitrile as the mobile phase (described in Section 2) were established for the HPLC fingerprinting and quantitative analyses of the herb.

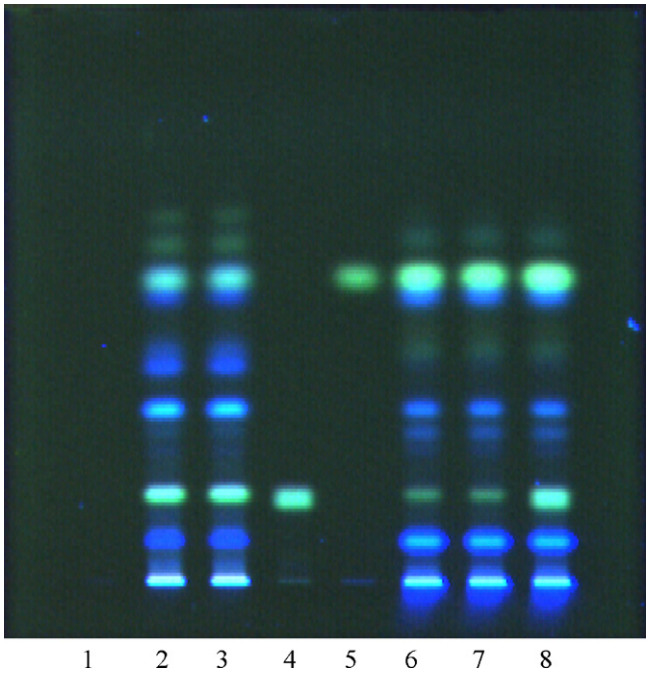

Fig. 1. HPTLC fingerprints of Rhizoma et Radix Notopterygii visualized under UV 366 nm R_f values of isoimperatorin and notopterol are 0.61 and 0.18, respectively; 1: solvent blank; 2: sample 1 (*Notopterygium incisum*); 3: sample 1 (duplicate); 4: notopterol; 5: isoimperatorin; 6: sample 2 (*Notopterygium forbesii*); 7: sample 2 (duplicate); 8: spiked sample 2.

3.3. HPLC fingerprinting identification

HPLC fingerprinting for Rhizoma et Radix Notopterygii was performed. The procedure for the extraction of crude drug and the HPLC separation conditions such as choices of mobile phase, gradient program and detection wavelength were optimized. Three peaks (1, 3 and 5) in the HPLC fingerprint profile were structurally identified as bergapten, notopterol and isoimperatorin, respectively, by employing LC–MS–MS (Table 1) and by comparing their retention times with those of reference standards. Fig. 2 shows a typical fingerprinting chromatogram of Rhizoma et Radix Notopterygii.

The HPLC fingerprints of 16 batches of samples from different regions of China were obtained under the optimized HPLC conditions. These herbs showed similar chromatographic profiles. After carefully analysing the fingerprint profiles of these samples, five common peaks with acceptable heights and good resolution were selected as characteristic peaks for the identification of the crude drugs originating from *N. incisum* Ting ex H.T. Chang and *N. forbesii* Boiss. Peak 5 (isoimperatorin) was selected as the marker peak. Relative retention times (RRTs)

Table 1
LC–MS–MS ions profile of three peaks in the HPLC fingerprint of Rhizoma et Radix Notopterygii

Peak	Identification	MS (*m*/*z*)	MS–MS (*m*/*z*)
1	Bergapten	217, 202, 174	202, 174
3	Notopterol	372, 203	337, 281, 269, 203, 187
5	Isoimperatorin	271, 203	203

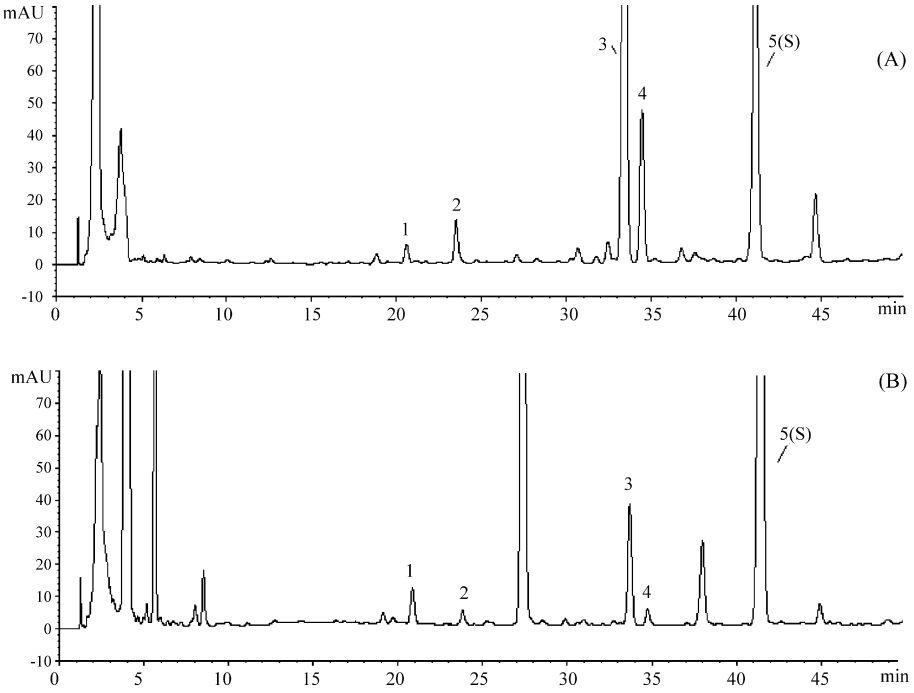

Fig. 2. Typical fingerprinting chromatogram of Rhizoma et Radix Notopterygii, (A) *Notopterygium incisum* and (B) *Notopterygium forbesii*, peak 1: bergapten; peak 3: notopterol; peak 5: isoimperatorin.

and relative peak areas (RPAs) of the five characteristic peaks were calculated as follows: RRT = retention time of characteristic peak/retention time of marker peak, and RPA = peak area of characteristic peak/peak area of marker peak.

The results from the 16 batches of samples (Table 2) indicated that the RPAs of the five characteristic peaks varied dramatically, but the RRTs were invariable for the herb and, thus, were a suitable parameter for identification. Therefore, a sample with a similar HPLC chromatographic profile and matched RRT values (Table 2) to the typical fingerprint chromatogram shown in Fig. 2 can be authenticated as genuine Rhizoma et Radix Notopterygii.

3.4. Validation of quantitative analysis

After carefully optimizing the HPLC conditions for the mobile phase, gradient program and detection wavelength,

Table 2
Relative retention times (RRTs) and relative peak areas of five characteristic peaks in HPLC fingerprints of 16 batches of Rhizoma et Radix Notopterygii

Batch numbers	Peak 1 (bergapten)		Peak 2		Peak 3 (notopterol)		Peak 4		Peak 5 (isoimperatorin)	
	RRT	RPA	RRT	RPA	RRT	RPA	RRT	RPA	RRT	RPA
1	0.501	0.046	0.571	0.090	0.812	0.443	0.837	0.859	1.000	1.000
2	0.501	0.034	0.571	0.060	0.812	1.768	0.837	0.402	1.000	1.000
3	0.501	0.034	0.571	0.109	0.812	0.630	0.837	0.812	1.000	1.000
4	0.501	0.036	0.571	0.038	0.812	0.102	0.837	0.820	1.000	1.000
5	0.501	0.023	0.572	0.072	0.812	1.605	0.838	0.375	1.000	1.000
6	0.501	0.023	0.572	0.035	0.812	1.199	0.838	0.179	1.000	1.000
7	0.501	0.026	0.572	0.082	0.812	1.213	0.838	0.196	1.000	1.000
8	0.501	0.032	0.572	0.079	0.812	1.605	0.838	0.294	1.000	1.000
9	0.501	0.029	0.571	0.164	0.812	1.263	0.838	0.176	1.000	1.000
10	0.500	0.032	0.571	0.107	0.812	1.142	0.838	0.180	1.000	1.000
11	0.491	0.021	0.564	0.011	0.811	1.039	0.836	0.083	1.000	1.000
12	0.491	0.022	0.564	0.008	0.811	0.205	0.837	0.025	1.000	1.000
13	0.491	0.014	0.564	0.002	0.811	0.023	0.837	0.003	1.000	1.000
14	0.490	0.007	0.564	0.002	0.811	0.019	0.836	0.003	1.000	1.000
15	0.491	0.029	0.564	0.003	0.811	0.095	0.836	0.011	1.000	1.000
16	0.500	0.025	0.570	0.068	0.811	0.837	0.837	0.146	1.000	1.000
Mean	0.498	0.027	0.569	0.058	0.812	0.824	0.837	0.285	1.000	1.000
S.D.	0.005	0.009	0.004	0.047	0.001	0.614	0.001	0.297	0.000	0.000
R.S.D. (%)	0.97	33.93	0.62	81.32	0.06	74.47	0.09	103.9	0.00	0.00

Note: Numbers 1–10 are samples of *Notopterygium incisum* Ting ex H.T. Chang and numbers 11–16 are samples of *Notopterygium forbesii* Boiss.

Table 3
Validation data for HPLC quantitative analysis of Rhizoma et Radix Notopterygii

Compound	Calibration equation	Correlation coefficient	Range of linearity (mg/L)	Precision R.S.D. (%)	Repeatability R.S.D. (%)	Recovery (%)	MDL (μg/g)	LOQ (μg/g)
Isoimperatorin	$Y = 36.61x + 51.79$	0.9998	5.0–200	0.67	1.8	98.5	1.2	6.0
Notopterol	$Y = 26.99x - 7.289$	0.9999	1.1–220	0.13	1.8	100.8	1.8	9.0
Bergapten	$Y = 43.75x - 6.984$	0.9999	0.56–22.4	0.20	1.4	98.3	1.4	7.0

Table 4
Influence of extraction time on extraction efficiency for three marker compounds ($n = 3$)

Extraction time (min)	Isoimperatorin (g/100 g)	Notopterol (g/100 g)	Bergapten (g/100 g)
30	0.24 ± 0.005	0.54 ± 0.007	0.018 ± 0.0005
60	0.26 ± 0.004	0.56 ± 0.007	0.020 ± 0.0005
90	0.25 ± 0.007	0.56 ± 0.008	0.020 ± 0.0004

Table 5
Contents of isoimperatorin, notopterol and bergapten in Rhizoma et Radix Notopterygii (*Notopterygium incisum* Ting ex H.T. Chang)

Batch number	Source	Isoimperatorin (g/100 g)	Notopterol (g/100 g)	Bergapten (g/100 g)
1	Hong Kong market	0.17	0.11	0.024
2	Hong Kong market	0.26	0.57	0.020
3	Hong Kong market	0.17	0.15	0.025
4	Hong Kong market	0.17	0.036	0.014
5	Pingwu, Sichuan Province, China	0.58	1.2	0.047
6	Tianzhu, Sichuan Province, China	0.71	1.1	0.036
7	Huzhu, Qinhai Province, China	0.79	1.2	0.075
8	Datong, Qinhai Province, China	0.62	1.3	0.058
9	Tongren, Qinhai Province, China	0.57	0.93	0.097
10	Hualong, Qinhai Province, China	0.63	0.92	0.076
	Mean	0.47	0.75	0.047
	S.D.	0.25	0.49	0.028

method validation for the quantitation of isoimperatorin, notopterol and bergapten was performed. The validation data are listed in Table 3. Satisfactory linearity, precision and repeatability were demonstrated. The minimum detection limit (MDL) and limit of quantitation (LOQ) are much lower than the contents of isoimperatorin, notopterol and bergapten present in the crude drug. These results indicated that the established method is suitable for the quantitative analysis of Rhizoma et Radix Notopterygii.

The variation in ultrasonic extraction yield with extraction time for the same batch of sample using methanol as the extraction solvent is shown in Table 4. A 60 min extraction ensured the quantitative extraction of all three marker compounds from the herbal samples.

3.5. HPLC quantitative analysis

Three marker compounds in 16 sample batches of Rhizoma et Radix Notopterygii collected from representative regions of China were quantitatively determined using the developed reverse-phase HPLC method. Each sample was analysed in duplicate to determine the mean contents of isoimperatorin, notopterol and bergapten in crude drug (Tables 5–7). It was observed that notopterol generally is more abundant in *N.*

Table 6
Contents of isoimperatorin, notopterol and bergapten in Rhizoma et Radix Notopterygii (*Notopterygium forbesii* Boiss)

Batch number	Source	Isoimperatorin (g/100 g)	Notopterol (g/100 g)	Bergapten (g/100 g)
1	Hong Kong market	0.37	0.54	0.014
2	Hong Kong market	1.4	0.038	0.026
3	Hong Kong market	0.66	0.023	0.013
4	Hong Kong market	0.61	0.021	0.008
5	Sichuan Province, China	1.2	0.19	0.046
6	Sichuan Province, China	0.98	0.31	0.042
	Mean	0.87	0.19	0.025
	S.D.	0.39	0.21	0.016

Table 7
Contents of isoimperatorin, notopterol and bergapten in Rhizoma et Radix Notopterygii from different sources

Contents (mean ± S.D.)	*Notopterygium incisum*		*Notopterygium forbesii*	
	Mainland China	Hong Kong	Mainland China	Hong Kong
Isoimperatorin (g/100 g)	0.65 ± 0.085	0.19 ± 0.045	1.09 ± 0.16	0.76 ± 0.45
Notopterol (g/100 g)	1.11 ± 0.16	0.22 ± 0.24	0.25 ± 0.085	0.16 ± 0.26
Bergapten (g/100 g)	0.06 ± 0.022	0.021 ± 0.005	0.044 ± 0.003	0.015 ± 0.008

incisum than in *N. forbesii*, whereas a lower content of isoimperatorin was observed in *N. incisum* than in *N. forbesii*. The mean level of isoimperatorin was less than that of notopterol in *N. incisum*, but in *N. forbesii* the mean level of isoimperatorin was greater than that of notoperol. It is worth noting that the amount of bergapten is far less than that of isoimperatorin or notopterol in the two species. The limited content of bergapten in Rhizoma et Radix Notopterygii suggests that bergapten is not suitable as a marker compound for the quality control of the herb. Both isoimperatorin and notopterol can be chosen as marker compounds to assess the quality of Rhizoma et Radix Notopterygii, and the two species (*N. incisum* and *N. forbesii*) can be differentiated through a comparison of the relative contents of isoimperatorin and notopterol [2,10].

The amounts of isoimperatorin, notopterol and bergapten in the two species of crude drugs from a Hong Kong market are remarkably less than those in drugs procured from Mainland China, probably due to regional differences and different methods of processing the herb.

4. Conclusion

A comprehensive quality assessment method for Rhizoma et Radix Notopterygii was established. In contrast to the conventional quality assessment standard of Rhizoma et Radix Notopterygii [1], the present improved method adopts HPTLC and HPLC fingerprinting of total extracts of the herb. This method provides more chemical information that can be used for the identification of the crude drug as well as for the quantitation of three pharmacologically active marker compounds that are directly associated with the quality of the herbal medicine. The HPTLC fingerprinting method has the advantages of simplicity, rapidity and visuality, whereas the HPLC fingerprinting method bears the advantages of specificity, powerful separation ability and ability to derive detailed chemical information. These two fingerprinting methods can improve the reliability of identification of Chinese herbal medicines. In the HPLC fingerprinting procedure, the RRTs of characteristic peaks were found to be an ideal quantitative parameter for the authentication of crude drugs. The pharmacologically active marker compounds isoimperatorin, notopterol and bergapten in the herbal medicine were quantitatively determined using a reverse-phase HPLC method that was developed and validated in-house. The results suggest that bergapten is not a suitable marker compound due to its low content in Rhizoma et Radix Notopterygii. Both isoimperatorin and notopterol can be used as marker compounds to assess the quality of this herb, and the two species (*N. incisum* and *N. forbesii*) can be differentiated through a comparative quantitation of isoimperatorin and notopterol.

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