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Simultaneous determination of betulin and betulinic acid in white birch bark using RP-HPLC

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Abstract

A simple procedure is described for the simultaneous extraction and determination of betulin and betulinic acid in white birch bark. The extraction was checked using different solvents: dichloromethane, ethyl acetate, acetone, chloroform, methanol and 95% ethanol (aqueous solution, v/v). It was found 95% ethanol was a good extraction solvent that allowed extraction of triterpenoid with a highest content. Separation was achieved on a reversed phase C_{18} column with acetonitrile–water 86:14 (v/v). Detection was accomplished with UV detection at $\lambda = 210$ nm. Using this method, the bioactive triterpenoid in white birch bark were simultaneously determined. Significant variations in the content of betulin and betulinic acid in white birch bark growing in different locations of China were also observed.

Keywords: White birch bark; Betulin; Betulinic acid; HPLC

1. Introduction

The white birch is widespread in the northern latitudes of the world, it represents an abundant and currently under-utilized natural resource, but the use of its bark is limited. It is only used as a low value fuel source in solid wood products industries. Betulin exists in large amounts in free form in the outer bark of white bark [1]. The abundant availability of betulin from natural resources gives rise to great research interest in its applications [2]. Betulinic acid was also isolated from whiter birch bark, and this triterpene acid has been the subject of intense studies because of its biological properties, especially its remarkable anti-melanoma and anti-HIV activities amongst others [3,4]. These two triterpenoid have similar molecular structures as shown in Fig. 1.

Although the isolation and identification of various triterpenes and triterpenoid saponines from birch bark have been reported by several groups [1,5], few quantification protocols have been described so far. To date, some analytical methods have been reported on the analysis of the various bioactive ingredients in the birch bark, including betulin, betulinic acid by HPLC or GC–MS. Analysis of betulin in *Grewia tiliaefolia* by HPTLC method has been described [6]. Betulinic acid has been determined by HPLC in *Doliocarpus schottianus* and by GC–MS after derivatisation in *Platanus acerifolia* and in *Betula pendula* [7,8]. The aim of this study was to develop a simple method for the simultaneous determination of betulin and betulinic acid in the white birch bark by HPLC with UV detection. Extraction with various solvent was also investigated during attempts to increase both betulin and betulinic acid concentration of extracts and to facilitate their detection using UV detector [9].

2. Materials and methods

2.1. Equipments

The analysis was performed using Shimadzu instruments (Shimadzu Corporation, Kyoto, Japan) consisting of a 20 μl injector loop, solvent delivery module (LC-10AD) with a double plunger reciprocating pump, DGU-4A degasser and an SPD-10A UV detector. Data was collected and processed using N2000 chromatography Data System (Zhejiang University Zhida Information and Technologies Corporation, Hangzhou, China).

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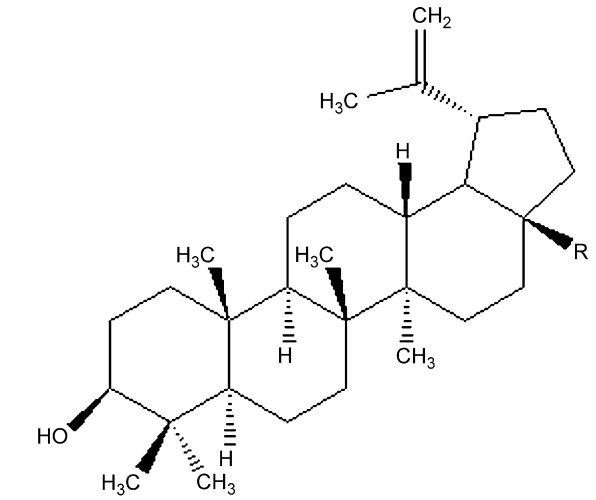

Fig. 1. Structure of betulin and betulinic acid in the extract of white birch bark.

2.2. Reagents and materials

2.2.1. Reagents and standard solutions

HPLC grade acetonitrile (E. Merck, Darmstadt, Germany) was used for the HPLC analysis. Deionized water was purified by Milli-Q system (Millipore, Bedford, MA, USA).

All other organic solvents used in this study were of analytical grade from Shanghai Chemical Reagent Corporation (Shanghai, China). Betulin standard was obtained from Sigma–Aldrich Chemical Corporation (USA). Betulinic acid was isolated from white birch bark, its chemical structure was confirmed by EI–MS, ^{1}H NMR and ^{13}C NMR and HPLC with its purity not less than 98%.

2.2.2. Plant material

The white birch (*Betula platyphylla suk.*) bark samples were collected in spring, 2006 from Liaoning Province (2006-04), Heilongjiang Province (2006-03), Jilin Province (2006-04) and Neimenggu Province (2006-03), respectively, in China. All the collected barks were immediately dried at 60 °C and stored in a dry and dark place.

2.3. Preparation of standard solutions

Standard solutions of betulin and betulinic acid were prepared from the pure product by dissolving appropriate weights in methanol, at concentration of 0.930 mg ml^{-1} for betulin, 0.428 mg ml^{-1} for betulinic acid and stored in refrigerator. Working solutions were prepared freshly every day by an appropriate dissolution of standard solution in methanol.

2.4. Preparation of sample solution

Accurately weighed equivalent to 20 g of dried bark pieces were refluxed with 200 ml dichloromethane, ethyl acetate, acetone, chloroform, methanol, 95% ethanol (aqueous solution, v/v), respectively, the mixture was extracted under a regressive cooler for 2 h, repeated for twice. After different solvent extract was colleted and filtered, the filtrate was dried under reduced pressure, respectively. Different dried extract was taken into a clean dry 50 ml flask, dissolved with methanol. The working concentration for the determination of assay of different solvent extract was approximately 1.0 mg ml^{-1}. All the volumetric flasks containing different solvent extract were tightly capped and stored at ambient temperature. After filtering through a 0.45 μm membrane filter, aliquots of 20 μl of clean solution were injected into the HPLC system, respectively.

2.5. Chromatographic conditions

Chromatographic analysis was carried out by Diamonsil C_{18} reversed-phase column (250 mm × 4.6 mm i.d.) with 5 μm pore size column (Dikma Technologies Corporation, Beijing, China). The mobile phase was acetonitrile–water (86:14, v/v). Betulin and betulinic acid were quantified by UV detector at λ = 210 nm [3]. Flow rate and injection volume were 1.0 ml min^{-1} and 20 μl, respectively. All chromatographic operations were carried out at ambient temperature. The chromatographic peaks of betulin and betulinic acid in different solvent extract were confirmed by comparing their retention time and UV spectra with betulin and betulinic acid standards. Quantification was carried out by the integration of the peak using external standard method. Each of the standard and the sample solutions were injected into the chromatograph and peak areas were recorded. From the peak area of betulin and betulinic acid the amounts in extract were computed by an external standard method.

3. Results and discussion

3.1. HPLC separation optimization

Previously, betulinic acid has been determined quantitatively in *Ziziphus fructus* by HPLC using a C_{18} column eluted with phosphate buffer:methanol and with UV detection at 210 nm [10]. In effect, the absorption of acetonitrile is lower than methanol, especially at the short wave such as 210 nm. During the short wave UV detection the minor noise and the high sensitivity make acetonitrile appropriate for HPLC analysis. In order to minimize background noise and to improve the detection limit, mobile phase was chosen from acetonitrile instead of methanol.

For HPLC analysis, initially various mobile phases were tried in attempts to obtain the best separation and resolution between betulin and betulinic acid. The mobile phase consisting of acetonitrile–water in the ratio 86:14 (v/v) was found to be an appropriate mobile phase allowing adequate separation of betulin and betulinic acid using Diamonsil C_{18} reversed-phase column at a flow rate of 1.0 ml/min. Under this system, the chromatogram of betulin and betulinic acid standard mixture is shown in Fig. 2.

It can be seen from Fig. 2 that a good separation can be achieved within 20 min using the conditions described. The retention time for betulin and betulinic acid was 13.3 and 16.5 min, respectively.

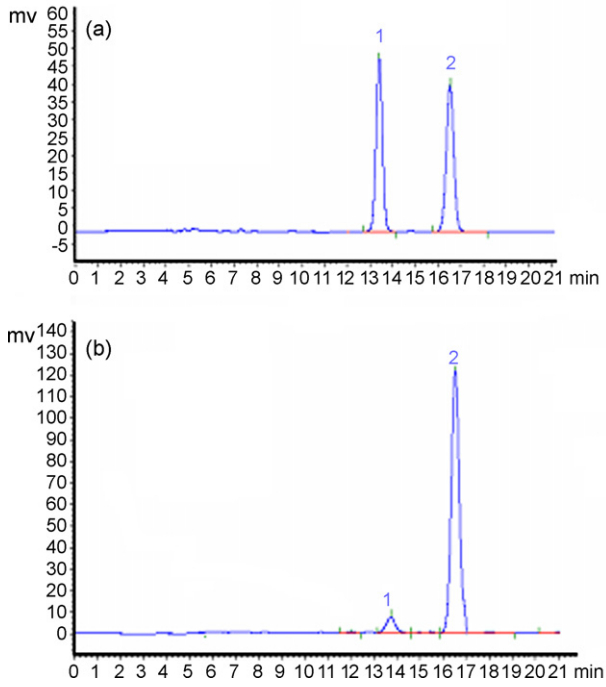

Fig. 2. Representative HPLC chromatograms of betulin and betulinic acid detected at 210 nm: (a) betulin and betulinic acid standard solution and (b) extract from white birch bark with 95% ethanol. Peak 1 for betulinic acid and peak 2 for betulin.

3.2. System suitability test

3.2.1. Linearity and detection limit

Linearity was checked by preparing standard solutions of both betulin and betulinic acid at five different concentration levels in the same volumetric flasks using their respective stock solutions. The calibration curves for betulin and betulinic acid were drawn in the concentration range of 0.093–0.930 and 0.0428–0.428 mg ml^{-1}, respectively. The spectrum was measured three times for each concentration. The equation for calibration curve of betulin is $Y = 235510x + 45588$ and for betulinic acid is $Y = 161543.3x + 9847.3$. The correlation coefficients of calibration plots were equal to 0.9995 and 0.9998, indicating good linearity in both cases.

Results of regression analysis on calibration curves and detection limits are presented in Table 1. The detection limits were evaluated on the basis of a signal-to-noise ratio of 3 (S/N = 3), the detection limits was 0.19 μg ml^{-1} for betulin and 0.31 μg ml^{-1} for betulinic acid.

3.2.2. Precision and stability

The measurements of intra- and inter-day variability were utilized to determine the precision of the developed assay method. A standard mixture solution of 0.171 mg ml^{-1} betulin and 0.285 mg ml^{-1} betulinic acid were prepared. The sample solution was analyzed in five times within the same day to determine the intra-day variability. The R.S.D. values were 1.31% for betulin and 1.05% for betulinic acid, respectively.

Table 2
Precision in the assay of betulin and betulinic acid by HPLC

Compound	Concentration (mg ml^{-1})	R.S.D. (%)	
		Intra-day	Inter-day
Betulin	0.171	1.31	1.05
Betulinic acid	0.285	1.65	1.33

Inter-day precision and accuracy of the method was tested for five times in two days at the same concentration levels. The R.S.D. values were 1.65% for betulin and 1.33% for betulinic acid, respectively (Table 2).

The stability of betulin and betulinic acid in methanol was determined for the samples stored at normal light conditions (tightly capped flasks kept on bench top). The samples were checked for 2 days of storage and the data were compared with freshly prepared samples. Solutions kept under normal light conditions were found stable and the R.S.D. values of assay were well below 2.0% against freshly prepared samples.

3.3. Sample analysis and recovery studies

3.3.1. Effect of extraction solvents

To compare extraction efficiency several solvents were evaluated, also extraction time and extraction times were optimized. The optimal experimental conditions were as follows: the white birch bark was extracted thrice (2 h each time) with solvent under reflux, and the ratio of the bark to the extraction solvent was 1:10.

This developed HPLC assay method was subsequently applied to a simultaneous determination of betulin and betulinic acid with different extraction solvents. Representative chromatograms of the standards and the solvent extracts from the white birch bark were shown in Fig. 2. The effects of the different extraction solvents (dichloromethane, ethyl acetate, acetone, chloroform, methanol and 95% ethanol) on the content of the compounds in the white birch bark extracts were studied (in Table 3).

The results showed that content of the compounds were strongly dependent on the solvents with different polarities. Ninety-five percent ethanol was a good extraction solvent that allowed extraction of triterpenoid with a highest content due to the co-solubility effect. Ethyl acetate and chloroform were the relatively better extraction solvents due to providing relatively higher yields compared with the other extraction solvents.

Table 1
Calibration curves and limits of the detection of betulin and betulinic acid in white birch bark

Compound	Retention time (min)	Standard curve	R^2	Test range (μg)	Limit of detection (ng)
Betulin	13.31	$Y = 235510x + 45588$	0.9995	1.86–18.60	3.8
Betulinic acid	16.46	$Y = 161543.3x + 9847.3$	0.9998	0.856–8.56	6.2

Y: peak area; x: concentration (μg); R: correlation coefficient regression equations; limit of detection: S/N = 3.

Table 3
Contents of triterpenoid in white birch bark with different extraction solvents

Extraction solvent	Betulin		Betulinic acid	
	Content ($n=5$, %)	R.S.D. (%)	Content ($n=5$, %)	R.S.D. (%)
Dichloromethane	12.20	2.58	1.08	1.85
Acetic ester	16.00	2.24	1.75	1.45
Acetone	13.02	1.91	1.51	2.43
Chloroform	17.00	1.56	1.33	1.25
Methanol	12.16	2.32	1.07	1.89
95% ethanol	20.22	2.25	1.86	2.38

Table 4
Recoveries of betulin and betulinic acid in the white birch bark ($n=5$)

Compound	Initial content (mg/g)	Amount added (mg/g)	Recovery (%)
Betulin	202.2	187.5	98.59
		94.3	97.27
Betulinic acid	18.6	19.2	99.57
		8.9	98.04

The extraction of methanol, dichloromethane and acetone were rather small. In all the six solvent extracts analyzed, betulin was found to be the most abundant component and the betulinic acid was rather low.

The recovery experiment of the betulin and betulinic acid were performed by adding betulin, betulinic acid standards to the white birch bark, which were treated according to the procedure described in Section 2.4 for five times. The recoveries for betulin and betulinic acid were between 97.27% and 99.57% (see Table 4).

3.3.2. Effect of white birch bark growing in different locations

The content of betulin and betulinic acid by HPLC were also analyzed for the white birch bark growing in different location. They were collected in spring, 2006 from Liaoning Province (2006-04), Heilongjiang Province (2006-03), Jilin Province (2006-04) and Neimenggu Province (2006-03), respectively, in China. All the collected barks were immediately dried at 60 °C and stored in a dry and dark place.

These dried barks were extracted as described in Section 2.4. The extraction solvent was chosen as 95% ethanol because betulin and betulinic acid could be extracted from white birch bark with a highest content. Results showed that all of the bioactive triterpenoid in white birch bark were strikingly dependent on the location (Table 5). The content of betulin and betulinic acid varied significantly in the white birch bark in different locations: the sample growing in Heilongjiang province had a higher content (betulin for 20.22%, betulinic acid for 1.86%) than all the other samples.

Table 5
Contents of betulin and betulic acid in white birch bark growing in different locations

Location	Lot no.	Betulin		Betulinic acid	
		Content ($n=5$, %)	R.S.D. (%)	Content ($n=5$, %)	R.S.D. (%)
Heilongjiang	2006-03	20.22	2.42	1.86	1.78
Liaoning	2006-04	11.3	1.56	1.22	2.21
Jilin	2006-04	16.1	1.78	1.42	1.85
Neimenggu	2006-03	14.22	1.56	1.35	2.01

4. Conclusions

The HPLC method mentioned here represented an excellent technique for simultaneous determination of betulin and betulinic acid in the extract of white birch bark, with good sensitivity, precision and reproducibility. The method gives a good resolution among betulin and betulinic acid with a short analysis time (20 min). Significant variations in the content of betulin and betulinic acid in white birch bark with different extraction solvents or white birch bark growing in different locations of China were observed.

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