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Journal of Pharmaceutical and Biomedical Analysis 45 (2007) 337–340
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

Separation and determination of closely related triterpenic acids by high performance thin-layer chromatography after iodine derivatization

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Received 28 February 2007; received in revised form 7 May 2007; accepted 9 May 2007
Available online 13 May 2007

Abstract

A method based on high performance thin-layer chromatography combined with densitometry for the simultaneous determination of oleanolic and ursolic acids is described. Because of the similarity of chemical structure the prechromatographic derivatization was necessary to separate these triterpenic acids. The samples were treated by 1% iodine solution in chloroform directly on the chromatographic plate and developed with the mobile phase consisting of A (petroleum ether), B (ethyl acetate) and C (acetone) (8.2:1.8:0.1, v/v/v). After drying, the plates were sprayed with 10% (v/v) ethanol solution of sulfuric acid(VI) and heated to 120 °C for 3 min. Quantification was performed in absorbance/transmittance mode at a wavelength of 530 nm by using a computer-controlled densitometer Desaga CD 60. The presented method was validated for linearity, precision and accuracy. Correlation coefficient ($r^2 > 0.99$), R.S.D. values (1.4–3.5%), detection limits as well as recovery values (98.4–103.1%) were found to be satisfactory.

The method has been successfully applied in the analysis of both triterpenic acids in plant extract.

Keywords: Oleanolic acid; Ursolic acid; HPTLC; Prechromatographic derivatization; Triterpenes

1. Introduction

Oleanolic and ursolic acid belong to the pentacyclic triterpenes. They often occur together in many medicinal plants and herbs [1].

They possess very important pharmacological properties. Numerous data on their anti-inflammatory, hepatoprotective, antitumour, anti-HIV, antimicrobial, antifungal, anti-ulcer, gastroprotective, hypoglycemic and antihyperlipidemic activity are reported in the literature [2–6]. They are relatively non-toxic and have been used in cosmetics and health products, e.g. oleanolic acid is marketed in China as an oral drug for human liver disorders and ursolic acid is used in antitumour therapy in Korean traditional medicine.

Several methods have been published for determination of triterpenes based on high performance liquid chromatography (HPLC) [7–13], micellar electrokinetic chromatography (MEC) [14,15] or gas chromatography [1]. Thin-layer chromatography has been also described [16–19] but oleanolic and ursolic acids are position isomers (Fig. 1) and their separation by TLC is rather difficult. There are some chromatographic systems to determine these triterpenic acids reported in literature but none of them enable their separation. On the other hand, modern TLC is powerful analytical technique, especially useful to analysis of plant material because large number of samples can be chromatographed simultaneously and the samples without any pretreatment can be applied. In case of compounds with similar chemical structure, sometimes the prechromatographic derivatization can be helpful in their determination. There are a lot of examples of use the specific chemical derivatization, for example, esterification was employed in analysis of primary, secondary and tertiary alcohols, hydrolysis (acidic or alkaline) was used in determination of flavonoids, triterpenes and cardenolide glycosides [20].

In this study the possibility of use the derivatization process to enable the separation of oleanolic and ursolic acids was investigated.

2. Experimental

2.1. Standard and sample preparation

All solvents and reagents were pro analysis grade from Polish Reagents (POCh, Gliwice, Poland). Triterpenic acids standards were purchased from Sigma (St. Louis, MO, USA).

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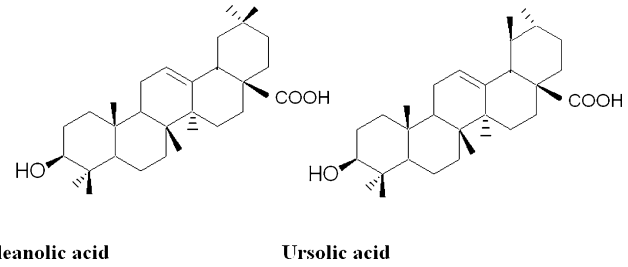
0731-7085/$ – see front matter © 2007 Published by Elsevier B.V.
doi:10.1016/j.jpba.2007.05.011



Oleanolic acid **Ursolic acid**

Fig. 1. The structures of ursolic and oleanolic acid.

Stock solutions of oleanolic and ursolic acid were prepared by dissolving 0.0200 g of each compound in 100 mL of acetone (final concentration: 200 μg/mL). Standard solutions at concentration of 40 μg/mL, 80 μg/mL, 120 μg/mL and 160 μg/mL were prepared by dilution of the stock solution with acetone.

To quantification 2.00 g of dry *Salvia officinalis herba* was extracted three times with 100 mL portions of diethyl ether within 12 h. The obtained extract was evaporated to dryness and the residue was dissolved in acetone to a fixed volume (100 mL).

2.2. Chromatographic conditions

In experiments HPTLC plates 10 cm × 10 cm or 10 cm × 20 cm coated with silica gel (Merck, Darmstadt, Germany) were used. The plates were washed with methanol and dried in a stream of hot air before use.

Five micro litres of standard solutions, 2 μL and 4 μL of sample of herbal extract were spotted using an automatic applicator Desaga AS 30 (Heidelberg, Germany) under nitrogen at 2.5 atm as streaks 6 mm long (track distance: 8 mm, distance from the left edge: 10 mm).

2.2.1. Prechromatographic derivatization

The plates were developed in horizontal Teflon DS chambers (Chromdes, Lublin, Poland) with 1% iodine solution in chloroform on a distance of 1.2 cm, and next the start zone was covered by a glass strip and the plates were placed in dark for 10 min. When the reaction was complete, the plates were dried in a stream of warm air to remove the excess of iodine.

2.2.2. Chromatography and determination

The plates were developed with a mixture of A (petroleum ether), B (ethyl acetate) and C (acetone) (8.2:1.8:0.1, v/v/v) as a mobile phase on a distance of 7 cm. After drying in a stream of warm air the plates were sprayed with 10% (v/v) H_2SO_4 in ethanol, dried for 10 min and then heated to 120 °C for 3 min.

The quantification was carried out by densitometric scanning (Desaga CD-60, Heidelberg, Germany) in absorbance/transmittance mode at $\lambda = 530$ nm (slit dimension: 4 mm × 1 mm).

Derivatization and determination were performed under controlled conditions (temperature 27 ± 2 °C, relative humidity 35–40%). The plates were scanned within 30 min; afterwards a progressive degradation was observed.

Documentation was obtained with use of a digital camera in daylight.

3. Result and discussion

Oleanolic and ursolic acids are important, natural compounds with confirmed pharmacological activity. They are common constituents of many medicinal herbs and plants. Thin-layer chromatography is an important tool of phytochemical investigations but the similarity of chemical structures of these triterpenic acids makes their TLC separation very difficult. In most cases these triterpenes are determined together as a sum [17].

Also our experiments on silica with use of mixtures of various organic solvents: heptane, cyclohexane, petroleum ether, toluene, dichloromethane, acetone, diisopropyl ether, diethyl ether, ethyl acetate, methanol, 2-propanol, butanol in various ratios, were not successful.

On the other hand, the prechromatographic derivatization process can improve the selectivity of separation substances with the same or similar chromatographic properties by exploiting their differing chemical behavior. In analysis of triterpenic acids esterification by use of acetyl chloride or oxidation by 2% chromium(VI) oxide solution in acetic acid have been employed [20,21] but obtained products had also similar chromatographic properties.

Prechromatographic derivatization with use of halogen (chlorine, bromine or iodine) has been employed in analysis of natural compounds, e.g. imperatorin, capsacinoids, steroids, indole alkaloids [19] but in determination of triterpenes this process was used for the first time.

The positive results were obtained after iodine derivatization. Oleanolic acid occurred more reactive and the reaction proceeded at room temperature (25–27 °C) whereas ursolic acid required higher temperature (above 40 °C). The derivatization conditions, e.g. concentration of iodine, time of reaction and mobile phase composition were chosen experimentally.

3.1. Method validation and sample analysis

The presented method was validated for linearity, precision, repeatability and accuracy. For proving linearity five calibration points were analyzed over the range of 0.2–1 μg of each compound. For each point, three measurements were made to

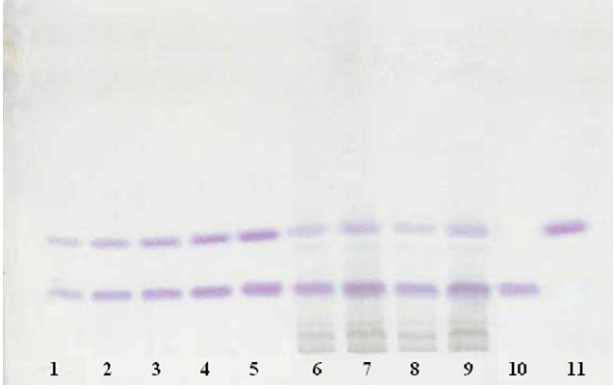


Fig. 2. The photograph of HPTLC plate: 1–5, calibration curve; 6–9, samples of extract of *folium Salviae*; 10, ursolic acid; 11, oleanolic acid. Documentation was obtained after derivatization with 10% (v/v) H_2SO_4 in daylight.

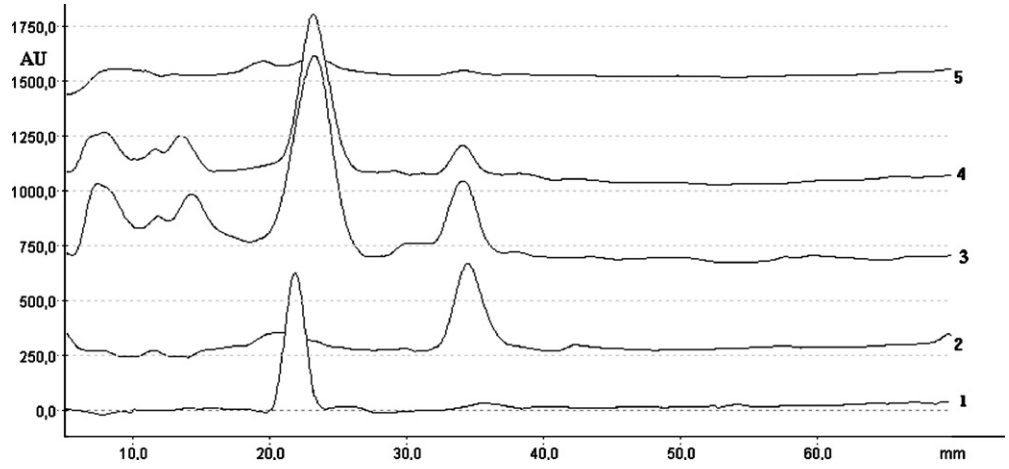


Fig. 3. Densitogram of HPTLC plate. The measurements were carried out at λ = 530 nm after derivatization with 10% (v/v) H_2SO_4 in ethanol. 1, standard of ursolic acid; 2, standard of oleanolic acid; 3, sample of extract of *folium Salviae* (4 μL); 4, sample of extract of *folium Salviae* (2 μL); 5, blank.

Table 1
Precision of the method

Ursolic acid			Oleanolic acid		
Amount (μg/spot)	Peak area (AU × mm)	%R.S.D.	Amount (μg/spot)	Peak area (AU × mm)	%R.S.D.
0.2	1584	3.5	0.2	1027	3.2
0.4	2512	3.4	0.4	1905	1.4
0.6	3426	1.4	0.6	2764	1.4
0.8	4236	2.3	0.8	3310	2.6
1.0	5243	2.3	1.0	4081	2.2

improve repeatability. The mean peak areas from ten different plates were taken for the construction of calibration curve. The data were analyzed by linear regression least square model and showed a good linear relationship over the tested range ($r^2 = 0.9992$ for ursolic acid and $r^2 = 0.9937$ for oleanolic acid). The linear regression equations for ursolic and oleanolic acids were $y = 4521x + 687.6$ and $y = 3756.5x + 363.5$, respectively.

The accuracy of the method was established by performing recovery experiments at two different levels. Known amount (0.0064 g) of each compound were dissolved in 100 mL of acetone. Five and 10 micro litres of solution were spotted and determined by the proposed method. Each solution was analyzed seven times. The %R.S.D. and recovery values are given in Tables 1 and 2.

In order to estimate the limit of detection (LOD) and quantification (LOQ), blank methanol was spotted three times following the procedure described in Section 2.2 and the signal-to-noise ratio was determined. The limits of detection were found 0.02 μg for ursolic acid and 0.05 μg for oleanolic acid. The limits of quantification were 0.07 μg and 0.17 μg, respectively.

Table 2
Accuracy of the method

Compound	Applied (μg)	Found ($n = 6$)	R.S.D. (%)	Recovery (%)
Ursolic acid	0.32	0.33	1.1	103.1
	0.64	0.63	2.8	98.4
Oleanolic acid	0.32	0.33	3.5	103.1
	0.64	0.66	3.4	103.1

The presented method was applied to determine the amount of oleanolic and ursolic acid in extract of *Salvia officinalis herba*. The identification of triterpenic acids was done on the basis of hR_f values (Fig. 2) and comparison of spectra. A good separation of oleanolic and ursolic acid were achieved (Fig. 3). The concentrations of ursolic and oleanolic acid found using the presented method were 9.3 mg/g and 5.0 mg/g of dry herb, respectively.

4. Conclusion

In the present study, an original and simple method for the separation of closely related isomeric triterpenic acid was presented. The method is based on different behavior of the investigated compounds after iodine derivatization.

The method was validated and successfully applied to simultaneous quantification of both triterpenes in herbal extract.

Acknowledgement

The research was supported by Ministry of Scientific Research and Information Technology, KBN No. 2P05F05028.

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