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ORIGINAL RESEARCH

Simultaneous Quantification of Galanthamine and Lycorine in *Galanthus fosteri* by HPLC-DAD

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ABSTRACT

Galanthamine and lycorine are known to possess important biological activities, widely distributed alkaloids in the members of Amarryllidaceae family. Also galanthamine is used for the treatment of mild to moderate Alzheimer's disease. In this study, the contents of these alkaloids have been quantitatively analyzed by high-performance liquid chromatography (HPLC) coupled with diode array detector (DAD) in the aerial parts and bulbs of *Galanthus fosteri* Baker collected from Akdag, Amasya during flowering and fruiting seasons. The chromatographic separation was performed using an isocratic system with a mobile phase of trifluoroacetic acid-water-acetonitrile (0.01:90:10, v/v/v) applied at a flow rate 1mL min^{-1} using diode array detector. A simple extraction method utilizing columns pre-packed with diatomaceous earth (Extrelut ®) was used. Validation procedures displayed that the method was specific, accurate and precise. Galanthamine was only detected in the aerial parts of the fruiting season with 0.0088 % average value. Also lycorine was only detected in the bulbs of flowering season with 0.0045 % average value.

Keywords: *Galanthus fosteri*, Amaryllidaceae, Galanthamine, Lycorine, HPLC-DAD.

Introduction

Galanthus L. species that belong to the family Amaryllidaceae, are represented by 14 taxa and one hybrid in Turkey (1). *Galanthus fosteri* Baker has natural distribution in south- and north-central of Turkey (1).

Plants of Amaryllidaceae family are well known for its contents of alkaloids with diverse chemical structures and a wide spectrum of biological activities (2). Galanthamine, the most important alkaloid found in Amaryllidaceae species, is used as an AChE inhibitor of herbal origin for the treatment of mild to moderate Alzheimer's disease due to its long acting, selective, reversible and competitive AChE inhibitor activity (3). Lycorine is also a common Amaryllidaceae alkaloid, and it has several biological activities such as antitumoral (4, 5), antimalarial (6), hepatoprotective (7-8), antiviral (9-10), antifungal (11), antiparasitic (12) and antiinflammatory (13) activities.

In the present study, the aerial parts and bulbs of *G. fosteri*, collected during flowering and fruiting periods, were investigated for their content of galanthamine and lycorine by using high-performance liquid chromatography (HPLC) coupled with a diode array detector (DAD). Moreover, in the context of validation procedures, the linearity, precision, limits of detection and quantification, accuracy, and specificity of the method were displayed.

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Experimental

Plant material

G. fosteri was collected from Akdag, Amasya on March 28, 2012 and April 1, 2013 during flowering and fruiting periods. The plants were identified by Prof. M. Ali Onur from the Department of Pharmacognosy, Faculty of Pharmacy, Ege University, Izmir, Turkey. Voucher samples of *G. fosteri* (No: 1516, 1525) are deposited in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Ege University.

Chemicals

Standard samples of lycorine and galanthamine were previously isolated in our laboratory and authenticated by spectral analysis (UV, IR, 1D NMR, MS) (14). TFA (trifluoroacetic acid) (Merck, 108178), HPLC grade acetonitrile (LabScan Analytical Sciences, LC 1005), and chromatographic grade double-distilled water were used for the HPLC analysis of the analytes and standards. Other chemicals were of analytical grade.

Sample preparation

The extraction procedure was carried out as previously, described (l5). Pre-packed columns based on diatomaceous earth for liquid–liquid sample purification were used for the extraction of samples. Dried and powdered plant material (200 mg) was macerated with 5 mL 2% hydrochloric acid for 5 h in an ultrasonic bath at 40°C and then the extract was made alkaline with 1 mL of 26% ammonium hydroxide, and the volume was adjusted to 10 mL in a volumetric flask with distilled water. After centrifugation at 5000 rpm for 10 min, aliquots of 3.0 mL were applied to the Extrelut® columns. The alkaloids were eluted with (5 mL × 3) chloroform after 10 min. The organic solvent was evaporated under vacuum to yield the alkaloidal extract. The extract was dissolved in 1 mL 0.1% TFA, filtered using a 0.45-µm filter (Grace Davison, USA) and injected (20 µL) into the HPLC column.

Chromatography

The analysis of the samples and validation studies were performed on a liquid chromatographic system (Agilent 1100 series), equipped with a quaternary pump, a vacuum degasser, a thermostatted column compartment, a manual injector with 20 µL loop (Rheodyne 7725i) and a diode array detector (DAD) (Agilent 1200 series). Column temperature was set at 25°C. The chromatographic separation was achieved isocratically using a mobile phase consisting of TFA–water–acetonitrile (0.01:90:10) on a Hichrom C18 column (5 µm, 250 mm, 4.6 mm), and detection was carried out at 290 nm. The flow rate was 1 mL min^{-1}, and the injection volume was 20 µL. The chromatographic run time was 45 min. Data analysis was performed using Agilent Chem Station software. All the calculations concerning the quantitative analysis were carried out by an external standard method based on peak areas.

Method Validation

Linearity, accuracy, and precision studies were carried out according to the ICH validation guidelines on the validation of analytical procedures (16).

Linearity

Standard stock solutions of galanthamine and lycorine were prepared by dissolving 5 mg in 10 mL 0.1 % TFA. These stock solutions were further diluted to five known concentrations in the range of 2.5 to 40 µg mL^{-1} and 1.25 to 20 µg mL^{-1} for galanthamine and lycorine, respectively. The linearity of the method was studied by injecting five known concentrations of the standards. Each standard solution (20 µL) was injected into the column in triplicate, and then the calibration curve of each analyte was obtained by plotting the peak area versus the concentration.

Precision

The precision of the method was assessed by studying intra-day and interday variation. Intra-day precision was determined by injecting solutions of five different concentrations of standard galanthamine (2.5, 5, 10, 20, 40 µg mL^{-1}) and lycorine (1.25, 2.5, 5, 10, 20 µg mL^{-1}) in triplicate on the same day. Inter-day precision was determined by performing the same procedure on two different days.

Limits of Detection (LOD) and Quantification (LOQ)

Limit of detection and limit of quantification were established at a signal to noise ratio (S/N) of 3 and 10, respectively. LOD and LOQ were experimentally determined by 10 injections of lycorine and galanthamine.

Recovery

The recovery of the method was performed by standard addition method. Three known amounts of individual standards were added to sample solutions from 0.0026 mg mL^{-1} to 0.0104 mg mL^{-1} at three spiked amounts and the mixtures were analyzed by the same method used in the analysis of lycorine and galanthamine in the plant samples.

Specificity

Specificity is described as the ability to measure the analyte response in the presence of components such as impurities, degredants, matrix, etc. (16). The specificity of the method for the analysis of lycorine and galanthamine was determined in the presence of other constituents found in the extracts.

Results and Discussion

Method Validation

Linearity

The calibration plots for galanthamine and lycorine were found to be linear within the range of 2.5 to 40 μg mL^{-1} and 1.25 to 20 μg mL^{-1}, respectively. The regression equation for galanthamine, was y = 12.685x + 1.3715 and for lycorine it was y = 15.194x + 1.7495. Excellent linearity was obtained for galanthamine (r^2 = 0.9998) and lycorine (r^2 = 0.9998) exhibiting a good correlation between the alkaloid concentration and the peak area.

Precision

The results of the precision analysis of galanthamine and lycorine summarized in Tables 1 and 2. These values were found to be less than 1.0 % for the two compounds indicating the good reproducibility of the method.

Table 1. Precision of galanthamine for *G. fosteri*

Amount (μg mL^{-1})	Intra-day precision (RSD, %)[a]	Inter-day precision (RSD, %)[a]
2.5	0.154	0.513
5	0.720	0.943
10	0.785	0.827
20	0.452	0.453
40	0.377	0.133

[a]Mean of three determination, RSD %, percentage relative standard deviation.

Table 2. Precision of lycorine for *G. fosteri*

Amount (μg mL^{-1})	Intra-day precision (RSD, %)[a]	Inter-day precision (RSD, %)[a]
2.5	0.154	0.513
5	0.720	0.943
10	0.785	0.827
20	0.452	0.453
40	0.377	0.133

[a]Mean of three determination, RSD %, percentage relative standard deviation.

Limits of Detection (LOD) and Quantification (LOQ)

The LOD was found to be 0.00982 μg mL^{-1}, and the LOQ was determined as 0.03675 μg mL^{-1} for galanthamine. The LOD and LOQ were calculated as 0.02472 μg $mL^{-1}$and 0.09247 μg mL^{-1} for lycorine, respectively.

Recovery

Recovery assay was carried out by spiking three different known concentrations of lycorine and galanthamine into sample solutions. The mean extraction recoveries were in the range of 97.959–101.023 % and 98.586-101.878 % for galanthamine and lycorine, respectively. The results of the experiments are given in Table 3.

Table 3. Statistical data for recoveries of galanthamine and lycorine

Analyte	Concentration in sample (mg mL^{-1})	Amount spiked (mg mL^{-1})	Mean Amount spiked (mg mL^{-1})	Mean recovery (%) ± SD	RSD (%)[a]
Galanthamine	0.00524	0.00260	0.00384	97.959 ± 0.074	0.945
		0.00520	0.00531	98.276 ± 0.097	0.894
		0.01040	0.00790	101.023 ± 0.843	0.843
Lycorine	0.00286	0.00140	0.00217	101.878 ± 0.037	0.907
		0.00280	0.00279	98.586 ± 0.041	0.768
		0.00560	0.00430	101.654 ± 0.072	0.894

[a]Mean of three determination, RSD %, percentage relative standard deviation.

Specificity

The acquisition of UV spectra with the DAD detector were used for the evaluation of the peak purity of galanthamine and lycorine. The spectra of the analyzed compounds are shown in Figures 1 and 2.

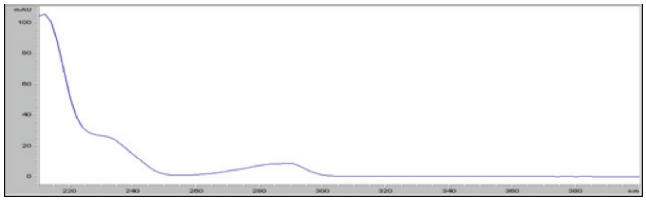

Fig. 1. UV spectrum of galanthamine

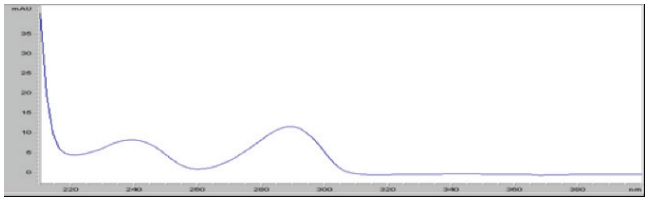

Fig. 2. UV spectrum of lycorine

Sample Analysis

Detection of these alkaloids was accomplished by comparison of the retention times and UV spectra with those of standard compounds (Figs 3 and 4). Under the described chromatographic conditions, galanthamine and lycorine were identified and quantified in *G. fosteri* specimens (Figs 5 and 6). Quantitative determination was carried out by the external standard method based on peak areas. The results of the mean values of three replicate injections of two compounds were reported in Table 4. Galanthamine was only detected in the aerial parts of fruiting season with 0.0088 % average value. Also lycorine was only detected in the bulbs of flowering season with 0.0045 % average value.

Table 4. Contents of galanthamine and lycorine in *G.fosteri*

G. fosteri (season)	Specimen	Content of galanthamine (%)	Content of lycorine (%)
Fruiting season	Bulbs	ND	ND
	Aerial parts	0.00882 ± 0.00025	ND
Flowering season	Bulbs	ND	0.00455 ± 0.00028
	Aerial parts	ND	ND

ND: not detected

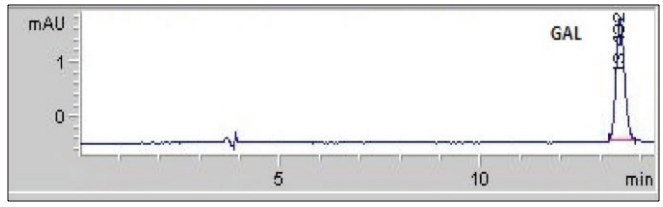

Fig. 3. HPLC chromatogram of standard galanthamine (GAL: Galanthamine)

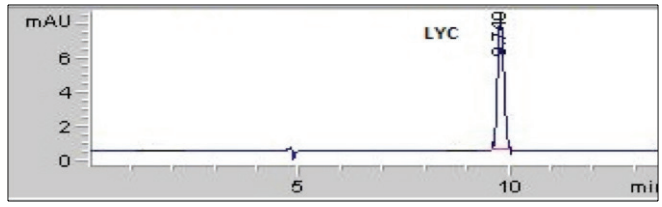

Fig. 4. HPLC chromatogram of standard lycorine (LYC: Lycorine)

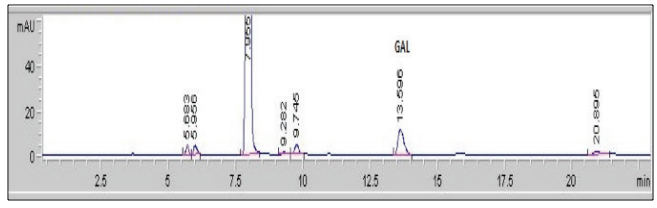

Fig. 5. HPLC chromatogram of the total alkaloidal extract of *G. fosteri* (Aerial parts of fruiting period)

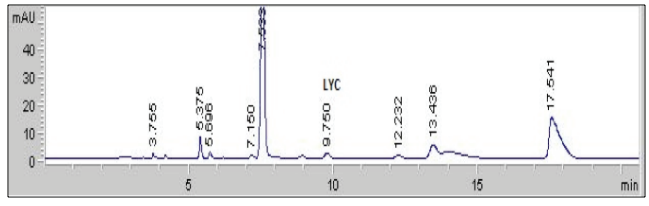

Fig. 6. HPLC chromatogram of the total alkaloidal extract of *G. fosteri* (Bulbs of flowering period)

Conclusion

In the present study simple, rapid, efficient and reliable method was applied for quantitative determination of galanthamine and lycorine in *G. fosteri*. Also the method was validated for linearity, precision, recovery and limits of detection and quantification. The datas of validation indicated that the method was appropriate for simultaneously determination of lycorine and galanthamine.

In our previous investigations, a number of *Galanthus* species has been found to contain lycorine, but not galanthamine (17-19). Lycorine and galanthamine have been simultaneously detected in *G. woronowii*, *G. cilicicus, G. elwesii* (15, 20, 21)

and in this study. Galanthamine was determined in the aerial parts of the plant collected in fruiting period whereas lycorine was detected in the bulbs of *G. fosteri* collected in flowering period.

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***Galanthus fosteri* Üzerinde Galantamin ve Likorinin YBSK-DAD ile Eşzamanlı Miktar Tayini**

ÖZ

Amaryllidaceae familyasının üyelerinde yaygın olarak bulunan galantamin ve likorin alkaloitleri, sahip oldukları önemli biyolojik aktivitelerle bilinirler. Ayrıca galantamin hafiften orta şiddete kadar olan Alzheimer hastalığının tedavisinde kullanılmaktadır. Bu çalışmada, bu alkaloitlerin, Amasya Akdağ'dan çiçekli ve meyveli dönemde toplanan *Galanthus fosteri* Baker bitkisinin toprak üstü kısımları ve soğanlarındaki içeriği, fotodiyot dizin dedektörlü (DAD) yüksek basınçlı sıvı kromatografisi (YBSK) ile kantitatif olarak analiz edildi. Kromatografik ayrım, mobil fazın trifloroasetikasit-su-asetonitril (0.01:90:10, v/v/v) olduğu, izokratik sistemde, dakikada 1 mL akış hızında, fotodiyot dizin detektörü kullanarak gerçekleştirildi. Önceden diyatomlu toprak ile doldurulmuş kolonların (Extrelut ®) kullanıldığı basit bir ekstraksiyon yöntemi kullanıldı. Validasyon işlemleri yöntemin spesifik, doğru ve duyarlı olduğunu gösterdi. Meyveli döneme ait toprak üstü kısımlarda % 0.0088 ortalama değer ile sadece galantamin tespit edilmiştir. Ayrıca çiçekli döneme ait soğanlı kısımlarda ise % 0.0045 ortalama değer ile sadece likorin tespit edilmiştir.

Anahtar kelimeler: *Galanthus fosteri*, Amaryllidaceae, Galantamin, Likorin, HPLC-DAD.

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