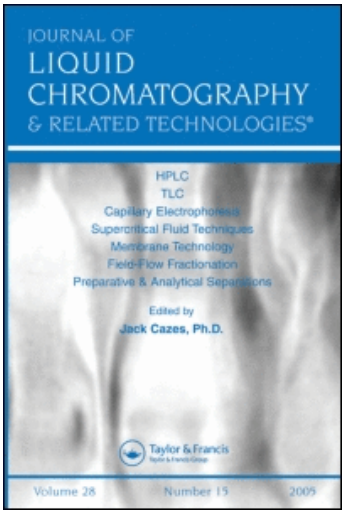

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QUANTITATIVE ANALYSIS OF THE ENANTIOMERIC NAPHTHAQUINONE DERIVATIVES FROM BORAGINACEOUS ROOTS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Some Boraginaceous roots contain red colored pigments, alkannin and shikonin, which are used in therapy as well as in food and textile industry. For the quantitative analysis of these enantiomeric naphthaquinone derivatives, a cellolose carbamate based chiral column (Chiralcel OD) on HPLC was used by using hexane/2-propanol (90:10) as mobile system. The method was applied to determine the alkannin and shikonin contents of the roots of eighteen Boraginaceous plants and results were also compared with those of the LiChrospher RP-18 column.

INTRODUCTION

"Havacýva" is the common name of *Alkanna tinctoria* Tausch. (Boraginaceae) in Anatolia. The root or root barks of the plant is used externally for the treatment of wounds and burns in folk medicine as well as red dyes for painting fabrics and carpets.[1] The same medical utilization is also described by Dioscorides, who lived in Anatolia, for the roots of *Anchusa tinctoria* (=*Alkanna tinctoria*) and *Echium italicum* in his book written 2000 years ago, revealing a utilization with a long history in Anatolia.[2]

Although, the name "havacýva" is mentioned only for *Alkanna tinctoria* in books and related documents on phytotherapy in Turkey,[1] during our field surveys throughout Anatolia, we have recorded that several other Boraginaceous plants are also called with the same Turkish name and usage.[9] These species and their localities are given below as a list:

Plant Name	**Locality**
Alkanna cappadocica Boiss.	Niðde, Merkez, Çarýklý
Alkanna megacarpa D.C.	Erzincan, Kemah, Çiðdemli
Arnebia densiflora (Nordm.) Ledeb.	Erzincan, Merkez, Bayýrbað
Echium italicum L.	Tokat, Merkez, Çerdiðin
Echium russicum J.F.Gmellin	Ardahan, Ölçek
Echium vulgare L.	Ardahan, Ölçek
Onosma sericeum Willd.	Erzurum, Ilýca, Tebrizcik
Onosma microcarpum Steven ex DC.	Erzurum, Horasan, Velibaba

This utilization for *A. tinctoria* root is not only limited to Anatolia, but is also recorded in some European documents as well.[3] On the other hand, in Oriental Medicine (Chinese and Japanese), the same utilization is described for the roots of another Boraginaceous plant, *Lithospermum erythrorhizon* Sieb.et Zucc.[4]

Though the wound healing effect of the roots is known since ancient times, the active principle was determined in 1978 as red naphthaquinone pigments.[3] Alkannin is the first identified member of this group of pigments and its dextrorotatory enantiomer which was isolated later was named as shikonin, referring to the Japanese name of *Lithospermum erythrorhizon* "shikon".

In addition to their wound healing activity, recently naphthaquinone pigments have gained attention as potential anticancer and antimicrobial agents. Moreover, as safe colorant materials these pigments are increasingly used for coloring foods, wines, cosmetics etc.[3] As a result of increasing demand

for these red pigments, Tabata et al.[5] developed a method for the mass production of shikonin using a plant cell culture technique from *Lithospermum erythrorhizon* cultures, and obtained up to eight times more pigment than that of the original plant.

Because of the stereoisomeric structure of shikonin and alkannin, the identification could only be done by circular dichroism spectral data.[6] For this reason, studies have been made for developing a rapid and easier method.

The first reported use of HPLC for the study of shikonin was performed by Fujita et al.[6] and several attempts have been made since than.[7] But all methods failed to give a full separation of shikonin and alkannin. Recently, Ikeda et al.[8] reported the use of a chiral phase HPLC for the separation of these isomers.

In this study, we aimed to develop a quantitative HPLC method by using the aforementioned[8] chiral separation method for determining the shikonin and alkannin content of the eighteen samples from the above listed plants, rapidly, selectively and precisely.

EXPERIMENTAL

Chemicals

Authentic samples of naphtaquinone derivatives were kindly supplied by Prof. M. Tabata and Prof. G. Honda (Kyoto University, Faculty of Pharmaceutical Sciences, Kyoto, Japan). HPLC grade solvents and bidistilled water were used for chromatographic studies. The mobil phases were degassed in an ultrasonic bath before being used. All chromatographic experiments were performed at room temperature.

Plant Materials

The research materials were selected according to the following criteria; (a). roots which are used in folk medicine (90C042, 90C137, 90E018, 90E021, 90E064, 90E165, 90E184, 94B142), (b). not used in folk medicine but contain visible red root barks (90C080, 90C149, 92A001, 94B005, 94B007, 94B101, 95B001), (c). not used in folk medicine, but has a reddish layer under the root bark which appear when scrapped (90A082, 90E222).

Table 1

Shikonin and Alkannin Percentages in Samples and Comparison of the Results in Two Different Column Systems

Species		Chiracel-OD			Lichro-Spher RP-18
Herbarium No. and Locality		Shikonin (%)	Alkannin (%)	Σ ratio (%)	Σ ratio (%)
Alkanna cappacocica Boiss					
90C137	Adana, Osmaniye, Hinzirli	0.033	0.25	0.283	0.286
92A001	Kayseri, Merkez, Erciyes	----	0.13	0.130	0.206
94B007	Kayseri, Develi, Gümüşören	0.091	0.63	0.721	0.661
94B101	Niğde, Bor, Kilavuzlu	0.070	0.65	0.720	0.626
94B142	Niğde, Merkez, Çarikli	0.043	0.34	0.383	0.327
Anchusa leptophylla Roemer & Schultes					
90E222	Erzurum, Naman, Yulkan Sivri	----	0.0058	0.0058	0.011
Anchusa undulata L. ssp. hybrida (Ten.)Coutinho					
90A082	Muğla, Göktepe, Ikizce	not detectable			
Echium italicum L.					
90C080	K. Maraş, Merkez, Andirin	----	0.12	0.120	0.19
90C149	Hatay, Kinkhan, Fevzi Paşa road	----	0.12	0.120	0.182
94B005	Kayseri, Merkez, Erciyes	----	0.073	0.073	0.123
95B001	Tokat, Merzifon, Samsun road	0.338	----	0.338	0.594
Echium russicum J. F. Gmellin					
90E165	Ardahan, Ölçek	----	0.06	0.06	0.190
Echium vulgare L.					
90E184	Ardahan, Ölçek	----	0.006	0.006	0.010

(continued)

Table 1 (Continued)

Species		Chiracel-OD			Lichro-Spher RP-18
Herbarium No. and Locality		**Shikonin (%)**	**Alkannin (%)**	**Σ ratio (%)**	**Σ ratio (%)**
Onosma sericeum Willd.					
90C042	K. Maraş, Cağlayancerit	0.447	0.041	0.488	0.402
90E018	Erzurum, Merkez, Taşligüney	0.172	----	0.172	0.166
90E021	Erzurum, Çat, near Bakimevi	not detectable			
Onosma microcarpum Steven ex D.C.					
90E064	Erzurum, Horasan, Velibaba	0.125	0.053	0.178	0.220
Arnebia sp. from herb dealer		16.83*	----	16.83	

* This value represents the ratio in root barks, while others in whole roots.

All specimens are deposited in the Herbarium of Gazi University, Faculty of Pharmacy (Ankara, Turkey). The materials, specimen numbers and their collection sites are described in Table 1.

Extraction

A piece of dried whole root is milled homogenously and 0.700 g is precisely weighed. The powdered material is then extracted two times with $CHCl_3$ (10 mL) overnight at room temperature. Combined extracts was evaporated *in vacuo*, dissolved in HPLC solvent mixture and diluted to 2.0 mL (Unhydrolysed Extract).

Alkaline Hydrolysis of Extract

0.700 g of precisely weighed material is extracted two times with $CHCl_3$ (10 mL) overnight at room temperature. Combined filtrates was evaporated *in vacuo* and dissolved in 2-propanol (0.1 mL) and hydrolyzed with 1 N NaOH

(2 mL) for 6 h at room temperature. The aqueous phase was then adjusted to pH 3 with 1 N HCl and extracted with $CHCl_3$ (2 mLx2). The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4 and evaporated to dryness *in vacuo*. The residue was dissolved in HPLC mobil phase and diluted to 2.0 mL.

Instrumentation

A Hewlett-Packard HPLC system was used consisting of the following components: Model 1050 pump, equipped with a Rheodyne Model 7125 valve fitted with a 20 µL loop, the model 1050 UV detector set at 520 nm and HP-3996A integrator.

Chromatographic Conditions

HPLC analysis was performed using two different type of columns:

Analysis by conventional reversed-phase HPLC

HPLC column:	LiChrospher 100 RP-18 (5 µm particle size; 4.mm i.d. x 250 mm, Hewlett-Packard Chemical Industries, Ltd)
Mobil phase:	Acetonitrile/water (65:35, v/v), containing trifluoroacetic acid (0.1%)
Flow rate:	1.5 ml/min
Detection:	UV, 520 nm

Analysis of enantiomers on chiral stationary phase

HPLC column:	Chiralcel OD (4.6mm i.d. x 250 mm, Daicel Chemical Industries, Ltd)
Mobil phase:	hexane/2-propanol (90:10, v/v)
Flow rate:	0.75 ml/min
Detection:	UV, 520 nm
Standards:	Alkannin, shikonin, acetylshikonin, deoxyshikonin, ß, ß-dimethylacrylshikonin, shikalkin

Calibration Graphs and Detection Limits

With Chiralcel column, calibration graphs for alkannin and shikonin were constructed by triplicate injection (20 µL) of the standard solutions at concentrations ranging from 50 µg/mL to 400 µg/mL (1.1 µg to 8.8 µg) for

alkannin and 85μg/mL to 680μ/mL (1.7 μg to 13.6 μg) for shikonin and plotting the peak areas versus the solute concentrations. At least five standard points were used for each graph and standard linear regression was used to determine the slope and intercept.

RESULTS AND DISCUSSION

Evaluation of the HPLC Method

A series of carboxylic acid esters of both stereoisomers, alkannin and shikonin, were isolated from the roots of Boraginaceae plants, including alkannan, acetyl-, isobutytyl-, ß,ß-dimethylacryl-, ß-hydroxy-isovaleryl-, isovaleryl-, α-methyl-n-butyl-, anhydro- and deoxy- derivatives. Among these derivatives alkannan, anhydroalkannin and deoxy-alkannin/-shikonin would not be hydrolysed when subjected to alkaline hydrolysis.

On the other hand, Ikeda et al.[8] proved that, during the alkaline treatment of samples for six hours, absolute configuration of both stereoisomers were not interchanged. The same statement was also reported by Chaisuksant et al.[10]

HPLC analysis of unhydrolysed samples on a chiral column revealed that there are no free, *i.e.* detectable, neither alkannin nor shikonin in all samples.

When test samples were applied on HPLC analysis by reversed phase column (LiChrospher 100 RP-18) after alkaline hydrolysis, alkannin and shikonin gave a single peak (t_R 4.695-5.120 min), *i.e.* the R and S stereoisomers could not be resolved by this method.

In addition, some peaks with shorter retention times were observed corresponding to unhydrolysable derivatives like deoxyshikonin, as compared with authentic specimen.

On the other hand, using a cellulose carbamate based chiral column, we observed a full separation of both enantiomers; shikonin is eluted between t_{R1} 22.4-24.5 min after injection, while alkannin eluted later (t_{R2} 28.0-30.8 min).

Quantitative analysis of shikonin and alkannin in each sample is performed by using both column systems.

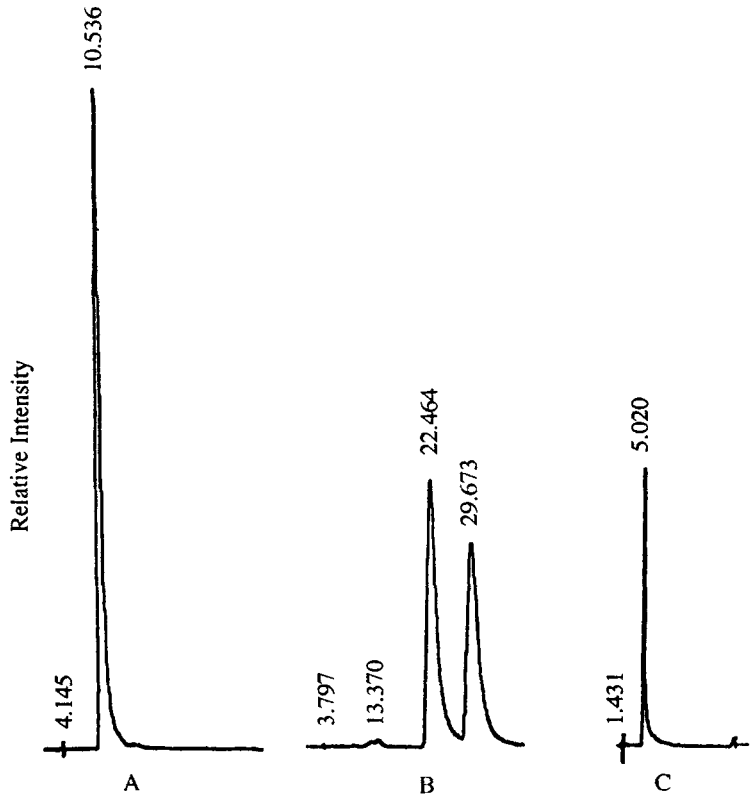

Figure 1. HPLC Chromatograms of *Onosma microcarpum* (90E064) Root Extracts: (A) Unhydrolyzed Extract on Chiralcel-OD Column, (B) Hydrolysed Extract on Chiralcel-OD Column, (C) Hydrolysed Extract on LiChrospher RP-18 Column.

Linearity

The detector response was linearly correlated with concentration, in the ranges 0.05-0.4 mg/mL for alkannin and 0.085-0.68 mg/mL for shikonin with Chiralcel OD column, and 0.085-0.68 mg/mL for total with LiChrospher RP-18 column. The regression equations and correlation coefficients determined for each standard were [y= 2846130.805x - 1150692.8] (r=0.996)(for alkannin), [y= 1706119.53x - 585125.96] (r=0.987) (for shikonin) in chiral column, and [y= 1432617.18x - 791628.38] (r=0.974)(for total), in reversed phase column, respectively. The sample concentrations were deduced by using the three equations.

Selectivity

Because of lack of placebo material and the difficulty of applying "standard addition method" for samples of different origin, selecitivity of the method was assessed by the following criteria. The unhydrolysed extracts did not give any peak at the retention times of alkannin and shikonin (Fig. 1).

After hydrolysis, the retention times of the appearing additional peaks were quite different from those of alkannin and shikonin. On the other hand, the extraction method also applied by Ikeda et al.[8] minimized the possible interfering compounds.

Precision (Repeatability)

The precision of the system was tested by 5 successive injections of standard solutions of alkannin and shikonin at 0.20 mg/mL and 0.22 mg/mL concentrations, respectively. The results expressed by the mean (X), S.D., and CV% are: X_{area}= 10 413 749±112 251 (1.08%) for alkannin, X_{area}= 5 929 833±56 108.7 (2.65%) for shikonin were satisfactory.

On the other hand, the method precision was assessed by triplicate determinations of 16 samples. As can be seen in Table 2, the repeatability expressed by X, S.D. and CV%, showed variations according to the origin of the material.

Evaluation of the Results

As shown in Table 1, highest naphtaquinone ratio is detected in the root barks of *Arnebia* sample (probably *Arnebia densiflora* (Nordmn.) Ledeb.) which is obtained from a herbdealer in Konya. Only shikonin is found in the root barks with a 16.83% ratio.

But this value is not comparable with those given for other specimens in the tables because, the *Arnebia* sample was packed as root barks and the result reflecting the ratio in root barks, while all other samples estimated from whole roots.

According to the results of analysis of 16 samples, alkannin and shikonin contents are not specific to genus even for species, but they change drastically according to the locality as well as plant to plant.

Table 2

Shikonin and Alkannin Amounts (μg/mL) in Samples on Chiral Column, with Standard Deviations

Species and Herbarium No.	Chiracel-OD Column (μg/mL) Shikonin (C.V.%)	Alkannin (C.V.%)	Shikonin: Alkannin ratio (%)
Alkanna cappadocca			
90C137	22.68±3.05 (13.45%)	171.50±8.65 (5.04%)	11.7:88.3
92A001	----	91.83±8.52 (9.27%)	0:100.0
94B007	80.65±16.62 (20.61%)	440.83±26.08 (5.91%)	15.5:84.5
94B101	48.70±4.45 (9.13%)	455.67±22.0 (4.84%)	9.7:90.3
94B142	30.00±8.48 (28.26%)	238.50±14.1 (5.91%)	11.2:88.8
Anchusa leptophylla			
90E222	----	0.29±0.03 (10.34%)	0:100.0
Echium italicum			
90C080	----	82.83±2.08 (2.53%)	0:100.0
90C149	----	85.67±1.53 (1.78%)	0:100.0
94B005	----	51.13±2.17 (4.24%)	0:100.0
95B001	236.33±14.93 (6.32%)	----	100.0:0
Echium russicum			
90E165	----	51.50±3.54 (6.87%)	0:100.0
Echium vulgare			
90E184	----	4.80±0.72 (15.0%)	0:100.0
Onosma sericeum			
90C042	312.75±32.51 (10.39%)	28.38±5.92 (20.86%)	91.7:8.3
90E018	41.50±4.25 (10.24%)	----	100.0:0
Onosma Microcarpum			
90E064	87.33±1.26 (1.44%)	41.75±2.47 (5.92%)	67.7:32.3
Arnebia sp.			
from herb dealer	11783±1020.21*		

* This value represents amount in the root barks, while others the whole root.

In the case of *Alkanna cappadocica* samples collected from different sites, all samples, except 92A001, contain a mixture of alkannin and shikonin, but alkannin is the dominant pigment. In two samples, 94B007, 94B101, the total ratio of both isomers is high, 0.712 and 0.720%, when compared to those of others. On the other hand, 92A001 contains only alkannin in low ratio.

Echium italicum samples also show variation, samples collected from neighboring cities in southern parts of Anatolia, K. Maraþ and Hatay, contains almost equal amounts of alkannin, but 94B005 collected from Kayseri, northern neighbor of K. Maraþ, contains only small amounts. The other sample obtained from far northern part, of Tokat, 95B001, has a completely different composition and ratio; 0.338% of shikonin, but not alkannin. In other *Echium* samples, *E. russicum* and *E. vulgare*, however, results agree in general with those of the previous data for *E. italicum*, contain only alkannin.

As for *Onosma* samples, the sample 90C042, is used as folk remedy and contains a high ratio of naphtaquinone pigments, 0.488%. The mixture is composed of alkannin and shikonin, but shikonin is dominant. On the other hand, sample 90E018, which is also used in folk medicine, contains only shikonin and the ratio is one third of the former. *O. microcarpum*, 90E064, contains both isomers, but shikonin is dominating.

Drugs containing naphtaquinone pigments are used in therapy for different purposes since ancient times. As a result of scientific studies, many of the effects are attributed to alkannin and shikonin derivatives; potent antibacterial and antifungal antibiotic activity,[3,11] antiinflammatory,[12,13] antiallergic,[3] antitumoral[3] and accelerator of tissue regeneration.[14] In addition, these compounds are increasingly used in cosmetics and foods. But all of these studies are performed without regarding the racemic structures. Recently, studies demonstrated that enantiomers of well known therapeutics have different pharmacodynamic and toxicological properties.[15] For this reason, researchers are investigating to isolate rasemics from mixtures to lower the side effects and increase the effects. For alkannin and shikonin, for the time being, which isomer is more potent to use in therapy and which is more safe to use as colorants in foods and cosmetics is not known. But recently, both enantiomers are reported to show anti-inflammatory activity in equal degrees by *in vivo* studies.[12] Thus it is important to identify and quantify the R and S forms separately, especially in the materials to be used orally. The conventional RP-HPLC method and other methods i.e. voltametric, based on total content of the isomers are not selective, as can be seen by comparison of the analysis results performed by two type of columns in Table 1, and does not reflect the real alkannin and shikonin content.

CONCLUSION

The HPLC method used by Ikeda et al.[8] for the enantiomeric separation of shikonin and alkannin, can be applied for the determination of both isomers in plants extracts, precisely and selectively. On the other hand, according to the results of this study, a statement which recorded in the previous documents that western, i.e. southern Europe and Turkey, Boraginaceae plants contains mainly alkannin derivatives, while eastern species, i.e. China and Japan, contain shikonin derivatives, may not be true, at least from the view point of Anatolian species.

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